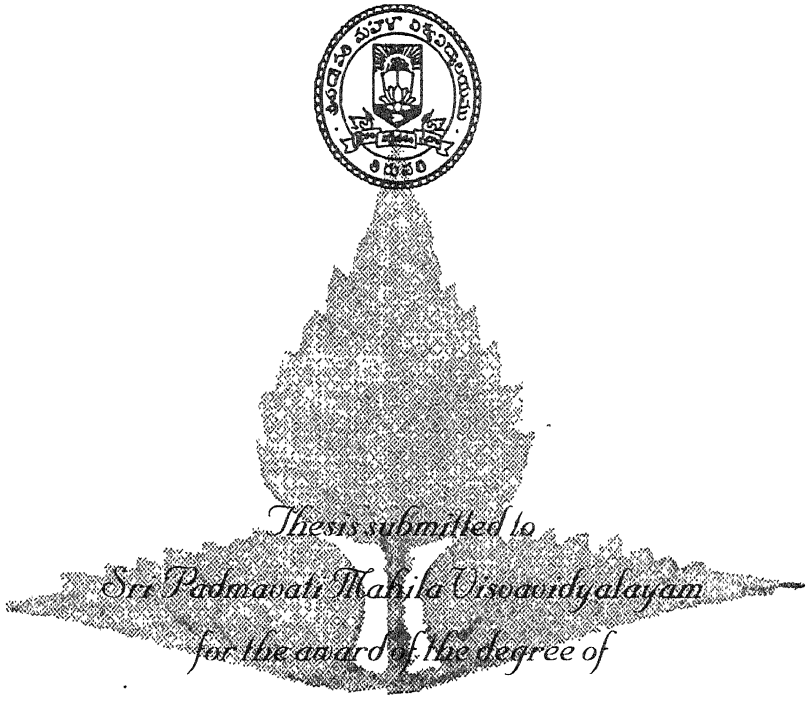
ASPECTS OF NEUROPHYSIOLOGY AND CARBOHYDRATE METABOLISM DURING 5TH INSTAR OF THE SILKWORM *Bombyx mori* L



Thesis submitted to
Sri Padmavati Mahila Visvavidyalayam
for the award of the degree of

DOCTOR OF PHILOSOPHY
IN
SERICULTURE

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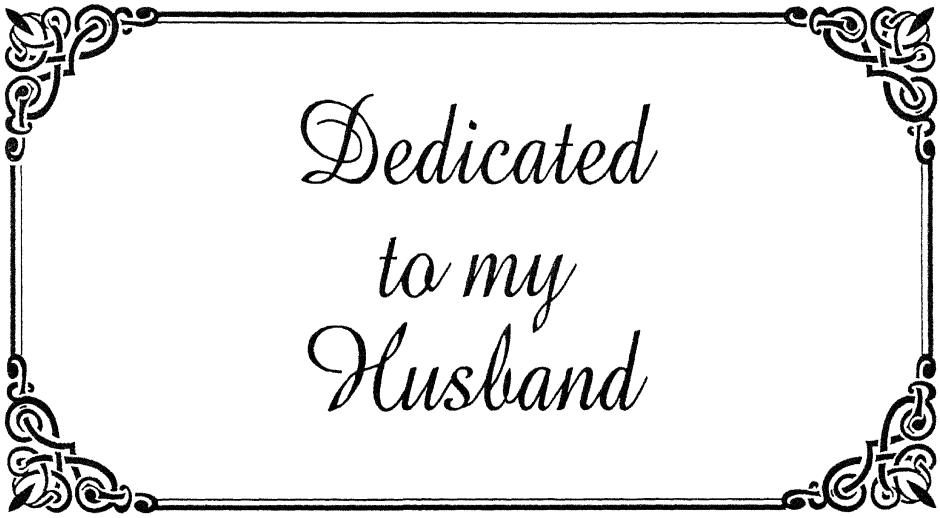
Dedicated
to my
Husband

CERTIFICATE

This is to certify that the thesis entitled **"ASPECTS OF NEUROPHYSIOLOGY AND CARBOHYDRATE METABOLISM DURING 5TH INSTAR OF THE SILKWORM *Bombyx mori* L"** *submitted by* **Mrs. P. JOSTHNA** *for the award of the Degree of Doctor of Philosophy in Sericulture is a bonafide record of research work done by the candidate during the period of her study under me, and that this thesis has not previously formed the basis for the award of any Degree or Diploma or Associateship or Fellowship or other similar titles.*

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Preface

Domestication of the silkworm *Bombyx mori* for obtaining silk to produce sophisticated, aristocratic, orthodox and exquisite textiles and dress material has been a part of the human civilization. The global silk industry has seen a radical change in the last two decades, both in terms of production and consumer preferences. Silk, being a protein fibre is close to human skin. It can absorb moisture up to 30% of its weight and make the user more comfortable. Economically too, mulberry cultivation and silkworm rearing leading to the production of silk do not consume much of fertilizers or insecticides and thus the process is more user-friendly.

Research on silkworms has been assuming importance in all countries. Sericulture research is being extensively undertaken in the southern states of India, especially in Karnataka and Andhra Pradesh. The main objective of research on silkworms is to achieve greater output of quality silk. In order to achieve this goal a thorough knowledge of all aspects of silkworm biology is necessary. The available information is more concentrated on silkworm nutrition, genetics, breeding and endocrinology. Limited information is available on the morphology, physiology, ecology and developmental biology of the silkworm. In the field of physiology most of the work done has been on tissues like silkgland and haemolymph and their role in silk production and other tissues such as the CNS and muscle have not received much attention. The intensive involvement of muscular activity vis-a-vis neural control of it in feeding, cocoon spinning and other activities is to be logically expected.

Studies on parameters which have special relevance to neural and neuromuscular activities would be of significant value in this regard. The present study was undertaken to examine the physiological and biochemical aspects pertaining to the nervous system, muscle and other tissues during the 5th instar of the silkworm *Bombyx mori*.

This thesis consists of three chapters. Day-to-day changes in biochemical parameters related to carbohydrate metabolism in haemolymph, silkgland, muscle and central nervous system of the 5th instar larva and also on the 1st day of the pupa are described in the first chapter. The second chapter deals with the extraction and estimation of biogenic amines during development and metamorphosis. The third chapter describes the changes in the spontaneous electrical activity of the nervous system. These three chapters are preceded by sections on the introduction and material and methods, and are followed by summary and conclusions, and bibliography.

Execution of the present investigation was an arduous task, owing to the limitations in maintaining a constant supply of the 5th instar larvae, conducting continuous day-to-day analysis in multiple replications for different parameters studied in different tissues etc. Effective use of the available facilities was made to complete this investigation in a satisfactory way.

The present study is expected to make useful contribution to the information on the biochemical and neurochemical changes taking place in the silkworm as the 5th instar larva prepares itself for the all important activity of the spinning of the cocoon.

ACKNOWLEDGEMENTS

I deem it a great privilege to express my gratefulnes to ***Dr. P. Murali Mohan,*** *Professor and Head, Department of Sericulture, Sri Padmavati Mahila Visvavidyalayam, Tirupati, for suggesting this problem for investigation, and for his illuminating guidance and encouragement. He played a key role at every stage of this work. My task has been facilitated by his kind care and patience, bringing the thesis to its present form.*

I sincerely thank the staff of the department of Sericulture, Sri Padmavati Mahila Visvavidyalayam, Tirupati for their help in various ways during this investigation.

I wish to express my thankfulness to my colleagues, friends and relatives who lent moral support at all stages of my academic pursuits.

I take this opportunity to place on record my indebtedness, love and affection to my parents ***Sri P. Dayal Naidu and Smt. P. Kusuma*** *and my brother* ***P. D. Brijesh Babu,*** *for their encouragement and moral support throughout my academic career.*

I acknowledge the cooperation and understanding of my juvenile daughter ***Manvitha*** *in allowing me to carry out this work.*

Finally, and most significantly, my heartfelt thanks are due to my husband ***Dr. C. V. Naidu,*** *Assistant Professor, Department of Biotechnology, S.V.University, Tirupati for motivating me to take up a research career and for his unstinted support throughout.*

I thank one and all who helped me in my work without which I could not have succeeded in my endeavour.

General Introduction

Sericulture is a labour-intensive agro-based industry, practiced all over the world for its commercial importance and employment potential. Because of its employment potential and profitability, low investment cost and high foreign exchange earnings, Sericulture could become an important factor in the economic development of India. It has become established as a cottage industry and the silkworm has attained great importance as an experimental tool. As an agro-based industry, Sericulture is now gaining popularity and getting expanded to almost all states in the country. Sericulture is a special type of land-based enterprise at the village level. Farm families attach great importance to this enterprise because of quick, periodic and high economic returns. The primary focus at the village level is on mulberry cultivation and silkworm rearing.

Silk is a natural fibre of animal origin which is unique because of its splendour, lustre and elegance, and is seldom challenged by other clothing materials. Hence it is regarded as "Queen of Textiles". It has been an inseparable part of Indian culture and tradition over thousands of years. Over the years the use of silk has been increasing and has become a part of the cultural life of the Indians.

Sericulture plays a vital role in the industrial sector. Silk is considered to be a potential raw material source for multidimensional applications of production and marketing. The silk products and byproducts of sericulture are useful in many ways. The utility of silk is high in textile industry, parachute industry, electrical manufacturing like insulation materials, artificial blood vessels, dental surgery, surgical sutures and bandages. It is quite interesting to learn that silk is used to make musical strings, tennis strings, strong ropes for mountaineering and fishing nets.

Not only silk but also silk waste is playing a dominant role in the economics of silk industry. The income rate of sericulturists has remarkably increased. Mulberry branches, silkworm litter and pupae are normally left over. These branches can be used as thin firewood, paper manufacturing and in pulp for manufacturing of rayon. Animals are fed with mulberry leaves and silkworm litter. They are also suitable materials for the manufacture of organic fertilizers. Swine or carps are extensively fed with silkworm pupae which contain fats and rich proteins. Since Sericulture has multidimensional applications and uses in various fields, it could draw the attention of scientists to evolve suitable conditions to develop Sericulture through several directions of research.

Sericulture in India

The Indian Sericulture industry achieved tremendous progress during the post-independence period, and the country could attain the status of the second largest producer of silk in the world. The achievement is not an overnight miracle but a result of incessant and relentless Research and Development (R & D) effort to evolve high yielding mulberry varieties and silkworm races, improved package of practices of Sericulture etc. The magnitude and employment potential of Sericulture can be appreciated from the fact that it is practiced in over 50,000 villages, providing gainful employment to about 59 lakh people, thus playing a pivotal role in poverty alleviation, employment generation and arrest of rural migration.

The major silk producing states in India are Karnataka, Andhra Pradesh, Tamil Nadu and West Bengal for mulberry silk; Bihar, Orissa and Madhya Pradesh for Tasar silk and Assam for Eri and Muga. Uttar Pradesh, Northeastern states, Rajasthan and Punjab are also practicing Sericulture. Karnataka is the largest silk-producing state with a share of nearly 60% of India's total production, with Andhra Pradesh standing the second.

There are many species of silkworms in India which produce cocoons of superior filament quality and larger quantity of silk. Among them *Bombyx mori* L of the family Bombycidae is the only species widely used for commercial rearing. *Antheraea assama, Antheraea mylitta* and *Philosamia ricini* of the family Saturniidae have also been commercially exploited.

The Silkworm, *Bombyx mori* L

Silkworm metamorphosis is a dynamic process and not a static phenomenon. From hatching to pupation the major behavioural function of the worm is feeding except during moulting and spinning. The voracious feeding of silkworm entails progressive changes in its structure and physiology.

The silkworm *Bombyx mori* L is a holometabolous insect, which undergoes a period of post-embryonic development which entails significant changes both in form and behaviour. The silkworm passes through 4 distinct stages in its life cycle, namely egg, larva, pupa and adult, the total duration of which is about 6 to 8 weeks. Larval stage is the most active period in its life cycle, which is divided into 5 distinct stages or instars. Each larval instar can be broadly divided into 2 phases. The feeding phase and the moulting phase. After feeding voraciously and having attained full growth for the

particular instar, the worm loses its appetite and the larva prepares to moult and cast off its old skin. Prior to each moulting the larva stops feeding and rests with its head held up.

After the silkworm passes through 4 moults, it reaches the 5th and final instar when it attains its maximum weight. At the end of 5th instar the silkworm builds a silken abode, viz the "Cocoon". It enters the pupal stage in this cocoon and remains in that stage for 8 to 14 days.

The pupal stage is a transitional phase during which significant changes take place. During this stage histolysis of the larval organs and histogenesis of the internal organs occurs actively. The larval organs that achieve histolysis are the silkgland, abdominal legs, ocelli, molting gland and the caudal horns. The mouth parts, thoracic legs, digestive organs, malpighian tubules, muscles and fat body exhibit extensive modifications as a result of histogenesis. During the pupal period reconstruction of the tissue takes place, involving particularly the eversion and growth of the wings and development of the flight muscles.

The adult moth emerges slitting through the pupal skin and piercing the fibrous cocoon shell with the aid of alkaline salivary secretion. The adult form is a typical insect, with a well marked head, thorax and abdomen and well developed wings, eyes, antennae, legs and reproductive organs.

Sericultural Research in India

Today's fast changing market is demanding high quality silk in large quantities. Years of research has contributed to the introduction of improved

versions of charkha, cottage basin and semi-automatic reeling devices which assure not only the quality but also the productivity and better working atmosphere. At the national level, the Central Silk Board (CSB), with a countrywide network of research and training centres, plays a pivotal role in R & D technology seeding and basic seed support. The states concentrate on extension and market support in the industry.

The silkworm is considered as an ideal experimental insect for the entomologists on account of its convenient body size, short life cycle and more number of generations per year. Extensive research has been carried out on various aspects of its biology viz biochemistry, physiology, anatomy, genetics and pathology. Silk being a proteinaceous fibre, biochemical research on silkworms assumes great significance both for quantitative and qualitative development of Sericulture industry.

All these years the silk industry has survived by following traditional methods of production. Time has come for the industry to shed old ideas and traditional practices and to adapt itself to the changing conditions. While much of the research in Sericulture is concentrated on nutritional improvement, evolving new races and devicing new methodology for effective silkworm rearing and cocoon production, it should be admitted that there is plenty of scope for research for productive Sericulture through physiological investigations. If this could eventually lead to the production of better cocoons, both in terms of quality and quantity, it could be an effective contribution to the silk industry.

Role of Nervous System

It is known that all facets of activity of an organism are controlled by the nervous system, either directly or indirectly. The spinning behaviour and the molting process of silkworm have an elaborate neural basis. If neural mechanisms underlying the spinning behaviour could be thoroughly analysed, it should be possible to effectively manipulate the spinning behaviour through the neural ferment. The central nervous system is perhaps a major controlling, directing and co-ordinating force for the entire metabolic machinery including silk production during the post-embryonic development of the silkworm. Hence it is necessary to study the anatomy, physiology and biochemistry of the neural and muscular systems in the 5th instar of silkworm and their bearing on silk production.

Physiological Research on Silkworm

In the silkworm the 5th instar is the last larval stage. This is the longest larval stage, entailing maximum food consumption. Hence its growth rate is also extremely high. It is during this stage that the silk-producing glands grow very actively. Significant physiological and biochemical changes occur during this instar.

Silkgland has been used as the experimental tissue in most of the studies, since it is an important organ which produces liquid silk as the source of cocoon fibre. The silk glands grow very fast, from the time of hatching to the final stage of mature larva. Studies were also performed on other tissues of the silkworm such as the central nervous system (CNS), muscle and haemolyamph.

Unlike the young larvae, the 5th instar larvae are susceptible to high temperature and high humidity. These environmental factors induce both physical and behavioural changes in the silkworm during different larval instars, with a qualitative and quantitative influence on silk production [Pant and Unni 1980]. Further, these factors play a significant role in the metabolism and life history of silkworms. [Rapusas and Gabriel 1976; Das 1984]. The effect of temperature and humidity on insect physiology, behaviour and development were reviewed by Shamshad Ali [1982].

Due to domestication from the times immemorial, the mulberry silkworm *Bombyx mori* has become delicate and is prone to attacks by a variety of parasites and pathogens. It has been observed that the Uzi fly preferentially infects the 4th and 5th instar larvae when they are sufficiently big in size [Jolly 1981]. Efforts have been made to study the effect of parasitization on the physiological aspects such as food digestability, growth and spinning [Srikanth *et al.* 1988]. Parasitisation of silkworm larvae by uzifly causes impairment of metabolic activities, resulting in the retardation of growth with faster maturation leading to early spinning [Venkata Rami Reddy *et al.* 1991].

Bombyx mori has also become an ideal model for researches on gene expression and regulation. The study of transgenic silkworm is important both in theory and practice. Transgenic approaches [Cheng *et al.* 1995] developed so far include microinjection, sperm carrier, Bm NPV mediation [Maeda 1985].

The metamorphosis of insects is under the precise control of two hormones, the juvenile hormone (JH) and 2-0-hydroxy ecdysone. Endocrinological studies over the years have greatly contributed to the elucidation of the functions and mechanisms of hormonal regulation in growth and development of the silkworm *Bombyx mori*. Recently, the use of vertebrate hormones to enhance the commercial characters of the mulberry silkworm *Bombyx mori* has taken a great turn. Singh and Datta [1980] reported that the administration of cyclic AMP and prostaglandin of the 5th instar larvae of *Bombyx mori* resulted in enhanced pupal and cocoon weights. Topical application of prolactin on *Bombyx mori* larvae accelerated the growth, shortened the larval duration and increased oviposition. [Bhaskar *et al.* 1983]. Bharathi *et al.* [1986] noted enhanced larval growth, early pupation and increased cocoon weight, following a pituitary extract treatment. Pushpa Rani [1997] reported the effect of selected vertebrate hormones on the growth and physiology of the silkworm *Bombyx mori* L.

Venkata Rami Reddy *et al.* [1994] for the first time identified vertebrate steroid like immunoreactive substances, i.e. testosterone, estradiol 17 ß and progesterone in the haemolymph of *Bombyx mori*. These authors opined that steroids in the silkworm might have some functions for the tissue biosynthetic activities.

Control of sex in mulberry silkworm is an important topic for sericulturists in theory and practice. As is well known, the male silkworm has higher viability than the female, and it consumes fewer mulberry leaves to produce the same amount of silk [Xia and Tanw 1980]. The silk yield of males

turns out to be 20% higher than that of females because the latter use more energy to produce eggs [Xiang *et al.* 1982]. Glycogen is stored in the eggs of the silkworm *Bombyx mori* and is made use of as and when energy is required during the course of diapause as well as embryonic development [Chino 1957].

Species-specific variations in the ontogenic pattern of various biochemical constituents are an essential feature of insect metamorphosis. The study of Chen [1971] provided an innovative report on the role of biochemical constituents during insect metamorphosis. Various biochemical constituents like total and soluble proteins, free amino acids, aminotransferases, protease activity etc have been examined in the tissues of silkworms with reference to its metamorphosis [Parenti *et al.* 1985; Siva Prasad and Murali Mohan 1990].

Proteins are the chief organic constituents of the cell. Both qualitative and quantitative studies on various aspects of insect protein metabolism, especially those during the post-embryonic development, have attracted the attention of several entomologists and biochemists [Poonia 1979; Anderson 1984; Venkata Reddy 1984].

Siva Prasad and Murali Mohan [1990] reported an increase in the levels of total and soluble proteins in *Bombyx mori* during metamorphosis. It has been demonstrated that an increase in the growth of the silkworm was accompanied by a similar increase in the synthesis of fibroin [Sakaguchi 1978; Venkata Reddy 1984].

Lipids constitute an essential component of all membranes and function as an important source of metabolic energy for cell maintenance, flight, reproduction, embryogenesis, and metamorphosis [Fast 1964; Gilbert 1967]. Work has been carried out on the metabolism of lipids and carbohydrates, their quantitative changes and their role during ontogeny and metamorphosis of insects [Wyatt 1967; Pant and Suman Kumar 1979]. Total lipids were analysed during different developmental stages in the silkworm *Bombyx mori* [Chinya and Ray 1976] and *Antheraea mylitta* [Agarwal *et al.* 1981].

Carbohydrates are important dietary constituents in most of the insects. During larval molts, total carbohydrates and glycogen decline by 50 to 60% and 80 to 90% respectively, while total lipids increase by 20%, indicating carbohydrates to be the predominant carbon source of chitin, a participant in energy metabolism as well as the substrate for lipid synthesis [Radha Pant 1984].

Perusal of the available literature reveals that little work has been done so far on day-to-day changes in different physiological and biochemical parameters during metamorphosis of the silkworm. The present investigation has been carried out on these aspects using day-to-day analyses of

carbohydrate metabolism during the 5th instar of *Bombyx mori* using tissues such as the CNS, muscle, silkgland and haemolymph in order to find a correlation with the spinning activity at the end of the 5th instar. In addition, day-to-day quantitative changes in some of the commonly occurring neurotransmitters were also examined in order to know if any of these transmitters has a correlation with the gradually increasing activity of the nervous system vis-a-vis the active state of the silkworm during the 5th instar, leading to the spinning phase. The possible involvement of these neurotransmitters in the activity of the nervous system was also explored by *in vitro* electrophysiological methods.

Material and General Methods

Test species

The present investigation was carried out on pure Mysore x NB_4D_2 (Multivoltine x Bivoltine) hybrid variety of the silkworm *Bombyx mori*. The experiments were carried out starting from the 1st day to the 7th (spinning) day of the 5th instar larva and the 1st day of the pupa. However, in cases where the 5th instar is extended to 8 days as it generally happens just before, during, and just after the summer, the experiments were conducted on the 8th day also. Since the experiments required a continuous supply of the required larvae, it was necessary to rear the larvae in the rearing house on the University campus, for ready supply whenever needed. The rearing process involved two activities, viz. mulberry cultivation and silkworm rearing.

Mulberry cultivation

The silkworm, being a monophagous insect, feeds exclusively on mulberry leaves. The mulberry plantation was maintained on the University campus. M_5 variety of mulberry plants were cultivated, the leaves of which are known to contain various nutrients in required concentrations for the growth of silkworms.

Silkworm rearing

Bombyx mori is a heterotherm and is a domesticated insect. It requires proper husbandry throughout its life cycle. Sufficient care was taken to provide the required hygiene and environmental conditions throughout the

rearing process. All the technical aspects involved in silkworm rearing were attended to with proper precaution. The silkworms were reared in large bamboo trays in the laboratory as per Krishna Swami [1978].

Rearing house

Silkworm rearing demands certain specified environmental conditions, particularly with reference to temperature and humidity. As such the silkworm larvae were reared in a separate rearing house, permitting enough light and ventilation under the normal 12 h light and 12h dark conditions. The room was reasonably made air-tight whenever necessary, in order to facilitate disinfection. The optimum temperature for normal growth in silkworm was between 24°C to 28°C. The silkworm rearing requires 70 to 85 percent relative humidity. Hence the larvae were reared during the period from August to March, avoiding the summer period of April to July.

Disinfection Procedure

Silkworms were reared with great care, since they are very susceptible to disease. To prevent disease, good sanitation methods and hygienic rearing techniques were followed. Before the commencement of each rearing the rearing room and appliances used for rearing were thoroughly washed with water, dried and disinfected with formalin. Formalin (2 to 4%) was sprayed on the equipment, walls, roof and floor of the rearing house in order to destroy the disease-causing organisms. The doors and windows of the room were then kept open at least for 24 hours before the commencement of rearing to get rid of the smell of formalin.

Incubation of eggs

Incubation aims at uniform development of the embryo, thereby securing uniform hatching through proper maintenance of the environmental conditions. Disease-free layings of the silkworm variety used were obtained from the central seed farm at Thondawada, a place near Tirupati. When the eggs reached the blue egg stage, the egg sheets were covered with a black paper to get uniform hatching. On the day of hatching, the eggs were exposed to bright light early in the morning.

Brushing

Brushing is the process of separation of newly hatched worms from the shells of the eggs and isolating them for convenient rearing.

After the hatching is completed, tender mulberry leaves chopped to approximately 0.5 cm^2 size, were sprinkled over the egg sheets, in order to facilitate the crawling of the larvae on to the mulberry leaves. After 15 to 20 minutes the sheet was turned upside down and the larvae along with the leaf were transferred on to a rearing sheet with the help of a feather.

Young age silkworm rearing

This is also called chawki worm rearing. The chawki worms were reared in large bamboo trays in which a paraffin paper was spread. Care was taken to prevent the Uzi fly and ant attack. Optimum spacing was produced to the worms in the trays to obtain maximum growth. Four feedings were given every day.

Late age silkworm rearing

The silkworms of the 3rd, 4th and 5th instar larval stages were considered as the late age worms. The 4th and 5th instars are the real feeding stages during which the worms consume 90-95 percent of the total feed and therefore it requires adequate spacing. The 2nd and 3rd instar larvae were fed with tender mulberry leaves, while the 4th and 5th instar larvae were fed with partially matured and coarser leaves. Five feedings were given per day to these larvae.

Bed cleaning

Cleaning of the silkworm bed is necessary to remove the excreta and leftover leaf. In the 1st instar, a single cleaning was given just before moulting. In the 2nd instar, two cleanings were given, one after the resumption of feeding and the other a day before the 2nd moult. In the 3rd instar, the cleaning was done three times and in the 4th and 5th instars, every day.

Moulting

The silkworm casts off its skin 4 times during its life period. This is called "moulting". Moulting is a very sensitive period in the life of the silkworm. At the time of each moulting, care was taken not to disturb the worms. Resumption of feeding was started only after all the worms have completely passed through the moulting, so as to ensure uniform growth of the larvae.

Healthy larvae of the 5th instar from the appropriate days were picked up for different experiments.

Statistical treatment of data

Statistical analysis of the data was performed as per Pillai and Sinha (1968).

a. Standard deviation was calculated with the following formula

$$SD = \frac{\sqrt{\Sigma x^2 - \frac{(\Sigma x)^2}{n}}}{n - 1}$$

Where Σx = individual observation

n = total number of observations.

b. Student's 't' test was calculated by using the following formula

$$t = \frac{m_1 - m_2}{\sqrt{\frac{SD\ 1^2 + SD\ 2^2}{(n_1 + n_2) - 2}}}$$

Where m_1 = the mean of first set of observations

m_2 = the mean of second set of observations

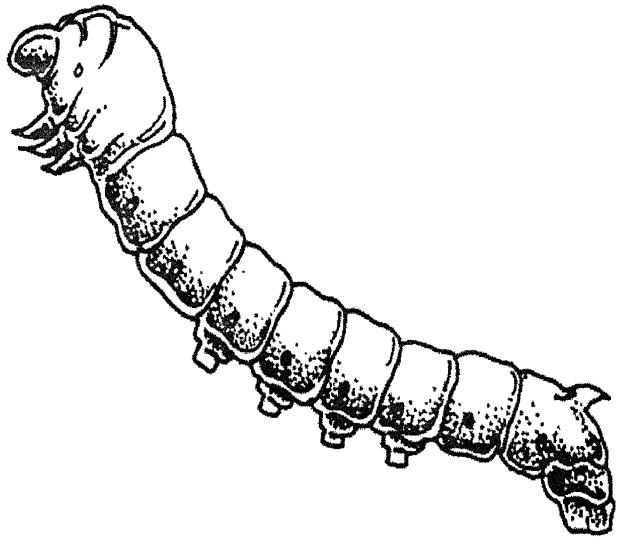
SD_1 = standard deviation of the first set of observations

SD_2 = Standard deviation of the second set of observations

n_1 = number of observations of the first set

n_2 = number of observations of the second set

Chapter - I



Aspects of Carbohydrate Metabolism

INTRODUCTION

Biochemical and physiological studies on silkworm have received considerable attention from various investigators [Horie *et al.* 1978; Horie and Watanabe 1983; Mathavan *et al.* 1984]. It has been demonstrated that increase in the body weight of silkworm is accompanied by alterations in the levels of various biochemical constituents like proteins, amino acids, carbohydrates and lipids and of activities of the enzymes like alanine aminotransferase [AlAT], aspartate aminotransferase [AAT], acetylcholinesterase (AChE) etc. [Church and Robertson 1966; Dhinakar 1988; Siva Prasad and Murali Mohan 1990].

Total Carbohydrates

Carbohydrates serve as the main source of energy and may be converted to fats for storage; they may also contribute to the production of amino acids. Consequently they often form an essential part of the diet and may be necessary in large amounts. However, carbohydrates are not always essential, and they can be replaced by proteins or fats. Larger amounts of nutrients, including the major types, may be stored in larval and adult fat bodies. This is the case, for instance, in lepidopterans like silkworms which do not feed as adults. Sufficient reserves are accumulated by the larva to supply to the adult metabolic processes. Similarly, if locusts are fed on grass during the first two larval instars, they can continue development up to the last larval instar without carbohydrates on the diet, because they have accumulated a sufficient quantity in the fatbody [Dadd 1963].

Carbohydrates are the predominant carbon source of chitin, a participant in energy metabolism and a substrate for lipid synthesis [Radha Pant 1984]. On the basis of qualitative changes in the levels of carbohydrates and lipids, Wyatt [1967] reported that insects convert fats into carbohydrates and *vice versa*. It is also well known that dietary carbohydrate-protein ratios influence the growth rates and that carbohydrate requirements often vary with age, sex and metamorphic stage [Beck 1956; Greenberg 1959]. Comprehensive reviews on carbohydrate metabolism in insects were given by Babers (1941), Yeager and Munson (1941) and Rockstein (1950).

The major function of carbohydrates in metabolism is to make available the fuel to be oxidised to provide energy for other metabolic processes [Martin *et al.* 1983]. In this role, carbohydrates are utilized by the cell mainly in the form of glucose [Harper 1985]. The silkworm growth and development depends on the physiology of the body. The physiology of silkworm has been shown to be dependent on the nutritional supply. The silkworm larval performance is dependent on nutritional value of the leaves [Rapusas and Gabriel 1976]. The carbohydrates and lipids are determining factors for the functions of the silkgland [Inagaki and Yamashita 1986]. The silkworm stores as bodily substance over 91% of the digested protein of the mulberry leaves, but it stores only 23% of the digested nitrogen-free extractives (carbohydrates) [Hiratsuka 1920], using the remainder for energy.

The carbohydrate content in insects is closely related to the physiological events such as the moulting process, metamorphosis, flight and diapause [Wyatt 1967]. Increased attention has been paid to tissue

interactions and regulatory mechanisms in carbohydrate metabolism in insects [Steele 1963; Murphy and Wyatt 1965; Wiens and Gilbert 1967; Goldsworthy 1970]. Kilby (1965) discussed the metabolisms of carbohydrates in the insect fatbody, while Sacktor (1965) has covered the aspects relating to muscle contraction. Florkin and Jeuniaux (1964) discussed the metabolism of carbohydrates in insect haemolymph. Urbani and Bellini (1959) observed very high utilization of carbohydrates for energy requirements in the embryos of mulberry silkworm, *Bombyx mori*. The haemolymph of starved locusts has a reduced concentration of total carbohydrates [Goldsworthy 1969].

Maheswaramma (1994) estimated total carbohydrates, glycogen and glucose in the tissues like Intestine, silkgland, muscle, haemolymph and fatbody of the silkworm *Bombyx mori* in different races and in different seasons.

Glycogen

Glycogen is a polysaccharide, forming the major energy reserve in many insects. Some glycogen may occur in most tissues, but commonly the main reserves are present in the fatbody. The main site of glycogen storage and synthesis is the insect fatbody [Babers 1941]. Glycogen has been reported in the midgut of several insects. It increases during digestion of food in larval tissues of *Bombyx mori* [Kuwana 1937] and decreases throughout the pupal life [Ludwig and Rothstein 1949].

Glycogen is known to play a major role in insect muscle contraction [Sacktor, 1965] as evidenced by the presence of phosphorylase activity in tissues of silkworm [Shigematsu 1956; Ito and Horie 1959]. Wigglesworth (1949) reported that glycogen gets deposited in the abdominal fatbody of *Drosophila*, with varying levels, during different stages of its life history. Glycogen has been observed in the tissues of the larval and pupal blowfly, *Phormia regina* [Stay 1959]. Yeager and Munson [1941] demonstrated that during the larval growth of the southern armyworm *Prodenia eridamia*, there was a large accumulation of glycogen in the haemocytes, which disappeared at the end of the pre-pupal stage.

The blood of *Bombyx mori* contains large amounts of anthrone-reactive material which was neither a reducing sugar, sucrose, nor glycogen [Wyatt *et al.* 1956]. Besides its role in energy production [Ludwing *et al.* 1965], glycogen is thought to be converted to other sugars that are used for synthetic purposes, such as ribose for RNA production [Sasse 1968], deoxyribose, chitin and AMPS [Van Der Starre - Van Der Molen - 1972].

Chadwick and Gilmour [1940] suggested that the energy for insect flight was supplied by the oxidation of carbohydrates stored chiefly as glycogen. This concept was supported by the study of Williams *et al.* [1943]. Glycogen is accumulated in *Calliphora* during larval development, most of which is utilized during metamorphosis [Hoglund 1976]. It has been reported absent from the blood of *Galleria* [Roy 1937] and bee larvae [Ronzoni and Bishop 1929]. Kuwana [1937] found a 10-fold increase in fermentable sugar after acid hydrolysis of *Bombyx* blood. Glycogen could be increased 6-fold in *Predenia* blood, by prolonged feeding of glucose [Babers 1941].

During the metamorphosis of many insects, as much as 73 to 100% of the stored glycogen may be utilized [Rockstein 1950], a process sometimes correlated with the corresponding increase in fat [Yeager and Munson 1941]. The high level of glycogen which occurs in the fat body of many insect species indicates the existence of an active pathway for glycogen synthesis, and the biochemical properties of the glycogen synthetase-complex have been investigated in several species under *in vitro* conditions [Vardanis 1963; Murphy and Wyatt 1965].

Glycogen Phosphorylase

The importance of phosphorylase as a control point in the metabolism of glycogen is related to the fact that activity of this enzyme can vary over a very wide range. A key control step in glycogenolysis is catalysis by glycogen phosphorylase, the regulation of which by a complex enzyme system has been intensively studied in vertebrate tissues [Fischer *et al.* 1971].

Phosphorylase exists in two interconvertible forms, phosphorylase 'a' and phosphophorylase 'b'. In the silkmoth fatbody, phosphorylase exists in active and inactive forms, which may be called fatbody phosphorylase 'a' and 'b' respectively, because of their functional similarity to the forms known in muscle [Stevenson and Wyatt 1964; Yanagawa and Horie 1977]. Glycogen is known to play a major role in insect muscle contraction [Sacktor 1965] as evidenced by the presence of phosphorylase activity in the tissues of silkworm [Ito and Horie 1959; Shigematsu 1956]. Contraction of muscle is accompanied by the conversion of phosphorylase 'b' to 'a' [Childress and Sacktor 1970; Achazi *et al.* 1975; Kallapur and Narasubhu 1976].

The conversion of phosphorylase 'b' to phosphorylase 'a' is catalysed by phosphorylase kinase [Wiens and Gilbert 1967a; Yanagava and Horie 1978]. The converted form can be activated by the action of cyclic AMP-dependent protein kinase, or directly activated by elevated intracellular free Ca^{2+} [Sogerling and Park 1974].

In intact silkmoth pupae, conversion of phosphorylase 'b' to phosphorylase 'a' accompanies stimulated glycogenolysis after wounding or chilling or during pharate adult development [Stevenson and Wyatt 1964; Wiens and Gilbert 1967a; Ziegler and Wyatt 1975]. Glucose inhibits fat body phosphorylase from *Locusta migratoria* [Applebaum and Schlesinger 1973] and *Samia cynthia* [Stevenson and Wyatt 1964] although it has a weak activating effect on rabbit muscle phosphorylase 'a' [Wang *et al.* 1965]. Conversion of phosphorylase 'b' to 'a' in homogenates of *Bombyx mori* larval fatbody requires ATP and Mg^{2+} [Yanagawa and Horie 1978]. They also made the interesting observation that addition of EDTA to homogenates caused the level of phosphorylase 'b' to fall almost to zero but without a stoichiometric increase in the amount of phosphorylase.

In the silkworm *Bombyx mori*, phosphorylase has been reported in the fatbody [Shigematsu 1956] and the midgut [Ito and Horie 1959], while in the developing embryo, phosphorylase activity has been shown to increase concomitantly with the utilization of glycogen [Shigematsu 1956]. In the cockroach, Steele (1963) reported activation of fatbody phosphorylase in response to a factor from corpora cardiaca, coinciding with the conversion of glycogen to trehalose.

Both the monohydroxyphenolic amine, octopamine and extracts of corpora cardiaca cause a marked depletion of glycogen levels in the nerve cord of *Periplaneta americana* [Steele 1963; Robertson and Steele 1973]. The glycogenolytic effect of both the corpora cardiaca factor and octopamine is due to the activation of phosphorylase [Robertson and Steele 1973; Hart and Steele 1973]. 5-HT (Serotonin) has been reported to have an effect on phosphorylase activity in the nerve cord of *Periplaneta americana*. In contrast to Octopamine, this amine in concentration as low as 0.1 mM reduces phosphorylase 'a' activity by as much as 28% [Hart and Steele 1969].

The glycogen phosphorylases of mammalian tissues have been extensively studied and found to undergo several types of activation which play important roles in the control of glycogenolysis [Brown and Cori 1961]. The phosphorylase of lobster muscle exhibits similar properties [Cowgill 1959].

Glucose

Glucose constitutes a major dietary constituent for the majority of insects, and its uptake from the gut would be greatly hindered by the occurrence of high concentrations of glucose in the haemolymph. The blood glucose level, on the other hand is negligible in comparison as it is usually below 30 µg/100 ml [Wyatt *et al.* 1956].

In the larva of the noctuid *Prodenia*, the blood glucose rises within 15 minutes of feeding; the glycogen in the fat body reaches its maximum 7 hours

after a meal [Evans 1932]. Glucose is rapidly converted to trehalose. Uridine diphosphate glucose, which in other organisms provides the glucose needed for glucoside synthesis, is present also in the fat body of *Schistocerca* and is utilized in providing the glucose for trehalose formation [Smith and Turbert 1961]. Treherne [1958] studied the absorption of glucose across the gut wall of the locust and reported that absorption was a process of facilitated diffusion, the necessary concentration gradients being maintained by the conversion of blood glucose into trehalose.

Nutritional tests based on longevity [Ito and Tanaka 1959] and utilization experiments [Horie 1960, 1961] indicated that some sugars such as glucose, fructose or sucrose have higher nutritional values than starch and dextrin for the silkworm. Not much of information is available on the participation of the glycolytic and pentose phosphate pathways in the catabolism of glucose in insects [Gilmour 1961; Chefurka 1965; Sacktor 1965].

Haemolymph glucose levels are elevated in response to various forms of stress and excersie [Willson and Rounds 1972; Matthews *et al.* 1976a], but no information is available concerning the origin of the extra glucose or the physiological mechanisms that regulate its production. Matthews *et al.* [1976 b] suggested that a major source of the additional glucose may be haemolymph-trehalose. Glycogen and glucose are generally present in haemolymph [Wyatt and Kalf 1957] and like trehalose, the levels of these sugars vary greatly among insect species.

Trehalose

Trehalose is a non-reducing disaccharide, derived from glucose-6-phosphate in the fatbody. It occurs in all insects, though not necessarily at all stages and it is commonly the most abundant sugar in haemolymph. Wyatt and Kalf [1957] reported that the major blood carbohydrate in insects is trehalose, that it is possibly an important reserve carbohydrate in insects. Fairbrain [1958] also reported the existence of trehalose in several other invertebrates.

Trehalose is the principal blood sugar in many species of insects including the silkworm *Bombyx mori* [Wyatt 1967]. The concentrations of glycogen and trehalose in insect tissues are related to physiological events, such as moulting, reproduction, flight etc. It was first shown in *Periplaneta americana* [Steele 1961] that the concentration of haemolymph trehalose increased while the glycogen content of the fatbody decreased. Trehalose is considered to be a metabolically active carbohydrate, because of its active biosynthesis in fatbodies of insects [Candy and Kilby 1961; Clegg and Evans 1961] and its rapid decrease during active movements of insects [Evans and Dethier 1957; Clegg and Evans 1961] or during starvation [Duchateau and Florkin 1959; Saito 1959; Horie 1961].

As trehalose is metabolised more, it is compensated for by production from glycogen, and so the glycogen reserves in a non-feeding insect steadily decline [Friedman 1978]. The haemolymph trehalose levels respond strikingly to nutritional state, quality and quantity of food intake [Hansen 1964]; to developmental stages of insects [Howden and Kilby 1960]; and to

physiological condition [Nowosielski and Patton 1964]. Recently it has been demonstrated that a non-reducing carbohydrate, which is present in the liquid silk of the *Bombyx* silkglands, liberates glucose on hydrolysis by an enzyme present in the silkgland cells [Shimada 1979]. The proportional decline in trehalose during adult development is thus less than that of glycogen, which falls by the time of emergence to one-fourth of its level in the pupae [Drilhon 1935].

Trehalase

Trehalase is one of the most important carbohydrolases in insects, occurring in the gut, flight muscles, fatbodies, labial glands, haemolymph and also in the silkgland of silkworms. It degrades trehalose to glucose for internal energy supply for the synthesis of chitin, muscular activity during flight, cocoon formation and other metabolic purposes. The enzyme trehalase catalyzes the hydrolysis of trehalose into 2 glucose molecules and is present in a number of organisms including bacteria [Hill and Sussman 1963], and yeast [Panek and Souza 1964].

Of the degradative pathway of trahalose, the presence of a hydrolytic enzyme, trehalase, was reported by many workers [Howden and Kilby 1956; Kalf and Rieder 1958; Friedman 1960; Saito 1960]. In silkworm larvae, trehalase was purified separately from muscle and the midgut, and its enzymatic properties were compared in order to consider the tissue specificity [Yanagawa 1971]. Trehalase plays a significant role in the supply of energy to an insect [Wyatt 1967] and the activity of trehalse midgut serves as an indicator of energy reserves resulting from availability of carbohydrate nutrients. High concentrations of trehalase during the moult and early stages

of Eri silkworm was reported by Chang (1964). Trehalase occurs in the muscles and the glucose produced is utilized as a fuel for flight by *Phormia, Locusta and Periplaneta* [Wyatt 1967].

METHODS

Estimations of the levels of different parameters of carbohydrate metabolism were carried out everyday during the 5th instar development and also on the 1st day of pupation. The haemolymph was collected by making an incision in the 1st pair of legs. After careful dissection, tissues such as the CNS, muscle and silkgland were isolated under cold conditions. Appropriate percent homogenates for the CNS, muscle and silkgland were prepared, while for haemolymph the necessary volume was taken depending upon the parameters studied.

The levels of total carbohydrates, glycogen, glycogen phosphorylase, glucose, trehalose and trehalase activity were estimated in the above tissues.

Total Carbohydrates

The total carbohydrate levels were estimated by the method of Carroll *et al.* [1956].

Homogenates (2%) of silkgland, muscle and central nervous system (CNS) were prepared in 10% trichloroacetic acid (TCA). In the case of haemolymph, 10 ml of TCA were added to 0.2 ml of haemolymph. They were then centrifuged at 1000 g for 15 minutes. To 0.1 ml of the supernatant, 5 ml of anthrone reagent were added and boiled for 15 minutes. The tubes were

cooled. The colour developed was read at 620 nm in a spectrophotometer [Elico, Hyderabad], using a reagent blank. The values were expressed as mg of glucose/g wet weight of the tissue or 1 ml of haemolymph.

Glucose

The Glucose levels were estimated by the method of Mendal *et al.* [1954].

Homogenates (2%) of silkgland, muscle and CNS were prepared in 90% methanol. In the case of haemolymph, 10 ml of methanol were added to 0.2 ml of haemolymph. The suspension was centrifuged and the supernatant containing glucose was decanted into a calibrated centrifuge tube. Powdered charcoal (10 mg) was added to this methanol extract. The charcoal does not absorb any hexose present but will remove organic substances which would otherwise interfere with the colour reaction. Methanol was then removed completely under reduced pressure while heating the tube in warm water. Deproteinising solution [5% TCA containing 0.1% silver sulphate] was added to the residual aqueous solution still containing the charcoal to bring the total volume to 5 ml. The tube was placed in a boiling water bath for 15 minutes and cooled. the suspension was centrifuged and the colour reaction was carried out with 1 ml of the clear supernatant as detailed below.

To 1 ml of clear supernatant, 3 ml of concentrated sulfuric acid were added in a wide test tube and mixed by vigorous shaking. The mixture was heated in a boiling water bath for exactly 6.5 minutes and subsequently cooled under running tap water. The intensity of pink colour developed was

read against the blank at 520 nm in a spectrophotometer. The glucose content was expressed as mg of glucose/g wet weight of tissue or 1 ml of haemolymph.

Glycogen

The glycogen levels were estimated by the method of Kemp and Van Heijningen (1954).

Since glycogen is insoluble in 80% methanol, its levels were estimated from the remaining precipitated residue after the extraction of glucose with methanol. The residue was suspended in 5 ml of deproteinising solution and the fluid level was marked on the centrifuge tube. The tube, covered with a glass cap was placed in a boiling water bath for 15 minutes. Then the tube was cooled under running tap water. The volume was made up to the mark with deproteinising solution in order to compensate its loss during evaporation, and then centrifuged.

The remaining procedure is the same as described under estimation of glucose. The glycogen content was expressed as mg glucose/g wet weight of tissue or 1 ml of haemolymph.

Estimation of Glycogen Phosphorylase [1,4-α-D-glycogen : ortho-phosphate α-D glycosyltransferase]

The activity levels of phosphorylase were assayed by the method of Cori *et al.* [1955] in the direction of glycogen synthesis by estimating the inorganic phosphate formed from glucose-1-phosphate [Fiske and Subba Raw 1925].

Homogenates (5%) of tissues prepared in an aqueous medium containing 0.1M sodium fluoride and 0.037M ethylenediamine tetra acetate [EDTA] at a pH of 6.5 as suggested by Guillary and Mommaerts [1962] in order to avoid enzymatic interconversion of the two phosphorylases, and 0.5 ml of haemolymph was used. The homogenates were centrifuged at 1000 g for 15 minutes. The supernatants were diluted four times with cysteine [0.03 M], ß-glycerophosphate [0.015 M] buffer (pH 6.5). The diluted supernatant (0.4 ml) was added to 0.2 ml of 2% glycogen and incubated for 20 minutes at 36°C. The reaction was initiated by adding 0.2 ml of glucose-1-phosphate [0.016 M] to one of the tubes for the estimation of active phosphorylase (a). To the other tube 0.2 ml of mixture of 0.016 M glucose-1-phosphate containing adenosine-5-monophosphate (0.004M) was added to estimate the total phosphorylase [ab]. After incubating for 15 minutes for total phosphorylase and for 30 minutes for active phosphorylase, the reaction was arrested by the addition of 1ml of 10% TCA and centrifuged. To the supernatant 1.0 ml of molybdate reagent was added followed by the addition of 0.4 ml of aminonapthosulphonic acid (ANSA) reagent. The contents were diluted to 10 ml with distilled water.

The intensity of colour was read within 5 minutes at 660 nm in a spectrophotometer against zero time controls. The enzyme activity was expressed as µ moles of inorganic phosphate formed/mg protein/h.

Trehalose

Trehalose in haemolymph was estimated according to the procedure of Saito (1963). Haemolymph was diluted 100 times in deionized distilled water. Trehalose level was spectrophotometrically determined by phenol-sulphuric acid method [Dubois *et al.* 1956] using glucose as the standard. The tissues (50mg) were homogenized in 1 ml of cold distilled water and centrifuged for 15 minutes at 4000 rpm. To 1 ml of diluted (5 times) supernatant, 0.5 ml of 5% redistilled phenol and 5 ml of concentrated sulphuric acid were added and shaken vigorously. The reaction mixture was then brought to room temperature and the orange-yellow coloured complex formed was measured at 490 nm in a spectrophotometer. The values were expressed as mg glucose/g wet weight of the tissue or 1 ml of haemolymph.

Trehalase Activity

Trehalase activity was determined by measuring the amount of reducing substance released from the substrate [Ishaaya and Swirski 1976].

Phosphate buffer (pH 6.5) (1 ml) was taken in a test tube and 0.5 ml of substrate (100m moles trehalose) was added. The mixture was incubated for 5 minutes at room temperature. The reaction was started by adding 0.5 ml of enzyme solution and continued for 30 minutes at 37°C. The reaction was stopped by adding 0.5 ml of DNS (dinitro salicylic acid) reagent and the mixture was boiled over a boiling water bath for 10 minutes. After cooling,

2.5 ml of distilled water was added and the absorbancy was measured at 540 nm in a spectrophotometer. The enzyme activity was expressed as mg glucose released/min/g wet wt of tissue.

VALIDITY OF EXPERIMENTAL PROCEDURES

1. Aliquots for assay

Aliquots were selected for the assay such that the initial rates were approximately as near as possible, yet providing sufficient product to fall in a convenient range for spectrophotometric measurement.

2. Enzyme units

Enzyme activities were expressed in standard units i.e. μ moles of product formed (or) substrate utilized per mg protein per hour (or) minute.

3. Substrate requirements

All the enzyme activity levels were determined at saturating substrate concentrations i.e. zero order.

4. Lambert-Beer Law

Almost all the products of the reactions were measured by using colorimetric procedures, in which the optical density (absorbance) of the resulting coloured complex was propotional to the concentration of the reaction products.

5. Enzyme nomenclature

The nomenclature of the enzymes followed in this thesis is according to the report of the commission on the enzymes of the international union of biochemistry [Pergamon Press, Oxford, 1966].

RESULTS

Day-to-day changes in slected parameters of carbohydrate metabolism were examined in four tissues, namely central nervous system (CNS), muscle, silkgland and haemolymph, on each of the seven days of the 5th instar and on the 1st day of the pupa. The results on all the biochemical parameters are given in Tables-1 to 7 and Figs. 1 to 28.

The 1st day of the 5th instar was taken as the reference point in the experiments, and the percent changes in all the biochemical parameters day-to-day as well as the statistical significance were calculated by taking this reference point as the control.

Total Carbohydrates

The results on total carbohydrates are presented in Table-1 and Figs. 1 to 4. Of the tissues examined, haemolymph recorded the highest level of total carbohydrate content on all days of the 5th instar. This was followed by the silkgland, muscle and CNS.

The total carbohydrate content showed a gradual increase in haemolymph, silkgland, muscle and CNS from the 1st day to the 7th day of the 5th instar. Thereafter they showed a sudden decline on the 8th day of the 5th instar and also on the 1st day of the pupa, when calculated taking the 1st day of the 5th instar as the control. However, the change in content from the 8th day of the 5th instar to the 1st day of the pupa was found to be less. Higher overall increases were noticed in the haemolymph followed by silkgland, muscle and CNS. On the 7th day of the 5th instar, when the overall increase in total carbohydrate content was calculated taking the 1st day of the 5th instar as the control, maximum increase was noticed in the CNS (+730.48%) followed by muscle (+504%) silkgland (+448.96%) and haemolymph (+258.59%). In all the tissues the changes were statistically significant from the 1st day of the 5th instar up to the 1st day of the pupa.

Glycogen

Glycogen levels examined in haemolymph, muscle, CNS and silkgland are presented in Table-2; Figs.5 to 8.

In general, muscle recorded a higher level of glycogen than the other tissues on all days of the 5th instar. In all the tissues which were examined in the present investigation the glycogen levels increased from the 1st day to the 6th day of the 5th instar and then decreased from 7th day of the 5th instar to 1st day of the pupa. Higher percent increases were noticed in the CNS (+938.72%) followed by muscle (+282.40%), silkgland (+171.70%) and haemolymph (+141.21%). On the 1st day of the pupa, greater percent decreases were recorded in the muscle (-82.99%) followed by silkgland (-56.10%), CNS (-53.75%) and haemolymph (-47.24%). The changes in the

levels of glycogen content were not statistically significant on the 2nd day for silkgland, 7th day for muscle, and 7th day of the 5th instar and 1st day of pupa for CNS. The changes on all other days for all the tissues were statistically significant.

Glycogen phosphorylase

The activity levels of glycogen phosphorylase 'a' and 'b' are presented in the Tables-3 and 4; Figs.9 to 12 and 13 to 16 respectively. The trends of change in these two enzyme activities contrasted each other.

The activity levels of phosphorylase 'a' showed a decreasing trend from the 1st day to the 5th day of the 5th instar in haemolymph and CNS. In the muscle and silkgland the activity levels of the enzyme decreased from the 1st day to the 6th day of the 5th instar. Thereafter the activity levels of phosphorylase 'a' showed elevation towards the end of the 5th instar and also on the the 1st day of the pupa in all the tissues. Maximum percent decrease was noticed in the CNS (-54.55%) followed by haemolymph (-44.94%), muscle (-43.17%) and silkgland (-42.83%). Maximum increase was noticed in CNS (+9.13%). The changes in the tissues were not statistically significant on the 2nd day of the 5th instar and 1st day of the pupa for muscle and silkgland, and on the 7th day of the 5th instar and 1st day of the pupa for CNS in CNS. The changes on all the remaining days were statistically significant.

The activity levels of phosphorylase 'b' showed an increasing trend from the 1st day to the 6th day of the 5th instar in silkgland, muscle and CNS.

While in the haemolymph, the activity levels of the enzyme increased from the 1st day to the 5th day of the 5th instar. Thereafter the activity levels slightly declined towards the end of the 5th instar and also on the 1st day of the pupa. Higher percent increases were recorded in the silkgland (+199.21%), followed by CNS (+156.88%), muscle (+143.40%) and haemolymph (+106.29%). Except the changes in the activity levels of phosphorylase 'b' on the 2nd day of the 5th instar in CNS, the changes on all other days of the 5th instar and also the 1st day of the pupa for all the tissues were statistically significant.

Glucose

Changes in glucose levels are presented in Table-5; Figs.17 to 20. The glucose content showed a gradual increase in haemolymph, silkgland, muscle and CNS from the 1st day to the 7th day of the 5th instar and a sudden decrease on the 1st day of the pupa. Higher overall increases were noticed in the haemolymph followed by muscle, silkgland and CNS. On the 7th day of the 5th instar, maximum percent increase was noticed in the silkgland (+445.01%) followed by muscle (+441.26%), CNS (+402.06%) and haemolymph (+216.63%), while in CNS maximum decrease was recorded on the 1st day of the pupa (-51.03%). The changes on all the days for all the tissues were statistically significant.

Trehalose

The variations of trehalose content in the muscle, silkgland, haemolymph and CNS are shown in Table-6; Figs.21 to 24. There was a continuous increase of trehalose content in all the tissues from the 1st day of the 5th instar and it reached the maximum on the 7th day of the 5th instar. A sudden decline was observed in all tissues on the 1st day of the pupa. Higher percent increase was recorded in the CNS (+5167.85%) followed by muscle (+3454.84%), silkgland (+1998.82%) and haemolymph (+1131.39%). In all the tissues the changes were statistically significant from the 1st day to the 7th day of the 5th instar and 1st day of the pupa.

Trehalase

The results on the activity levels of trehalase are shown in Table-7; Figs.25 to 28. The trehalase activity showed a decreasing trend from the 1st day to the middle of the 5th instar and then showed elevation towards the end of the 5th instar and also on the 1st day of the pupa in haemolymph, CNS, muscle and silkgland. Higher percent increases were noticed on the 1st day of the pupa in the silkgland (+148.27%) followed by CNS (+97.61%), haemolymph (+38.74%) and muscle (+13.35%). Higher percent decreases were recorded on the 5th day of the 5th instar in the haemolymph (-91.00%) followed by silkgland (-85.53%) and muscle (-48.54%). Higher trehalase activity was recorded in muscle among the tissues examined. The changes on all the days in all the tissues were statistically significant except on the 2nd and 7th day of the 5th instar in the CNS.

DISCUSSION

Total Carbohydrates

Insects need carbohydrates as the major fuel reserve for their growth and development. Holometabolous insects like silkworms consume sufficient quantity of food, for energy during the active larval life and to reserve energy for the pupal and adult non-feeding stages. Carbohydrate is the basis of entire biological activity. The large carbohydrate molecules are degraded to their component units of simple sugars, which then are in the form in which they are most readily available as a source of energy for growth and development of insects. The carbohydrates enter into chemical reactions, releasing energy which becomes available for a multitude of reactions.

The silkworm consumes mulberry leaves, which contain carbohydrates, proteins and other metabolic substances for energy. They are digested by the respective enzymes into micromolecules which are absorbed into the midgut epithelium. Through the haemolymph these substances are distributed to different parts of the body for cellular metabolism, wherein the micromolecules get reconverted into complex macromolecules like proteins, carbohydrates and lipids characteristic of the animal.

The present investigation deals with the levels of total carbohydrates in the tissues like CNS, muscle, silkgland and haemolymph of the silkworm, *Bombyx mori*. The relative concentrations of total carbohydrates in all the tissues of silkworm show that the haemolymph contains a higher level of

carbohydrates followed by the silkgland, muscle and CNS. The levels of total carbohydrates increased in all the tissues from the 1st day to the 7th day of the 5th instar and thereafter decreased on the 8th day and also on the 1st day of the pupa.

Higher level of carbohydrates during early part of the 5th instar and a decrease thereafter towards the end indicates the differential physiological status at different stages during the 5th instar. Lower values towards the end 2of the 5th instar may be attributed to the rapid utilization for spinning activity and conversion for synthesis of other body constituents such as proteins (including the silk proteins) and lipids (fuel reserve) for all the physiological activities during the non-feeding stages that follow. Of the total carbohydrates derived, about 20% are utilized for lipid synthesis during larval ecdysis while the remainder fulfills the energy needs and serves as a substrate for chitin synthesis. On commencement of spinning, the carbohydrates are probably utilized for energy for silk spinning, leading to their depletion during this period. This is in agreement with the observations of Bade and Wyatt (1962).

In *Bombyx mori*, the 5th stage alone consumes about 87.67% of the total food intake for the entire larval life [Matsumura and Takeuchi 1950]. The total body weight rises steadily during larval life until a day or two before the start of spinning, then falls precipitously during the spinning period to half its maximum value [Heller and Sweichowska 1948].

In haemolymph the total carbohydrate content progressively increased from the 1st day to the 7th day of the 5th instar and thereafter decreased on the 8th day and also on the 1st day of the pupa. Haemolymph is an extracellular fluid in silkworm as in other insects. The physiological function of the blood is to transport nutrients and waste products. Thus changes in the composition of haemolymph reflect the morphogenetic and biochemical transformations taking place in the insect tissues [Pawar and Ramakrishnan 1977]. The presence and increase of carbohydrates in haemolymph is attributable to their release from other tissues. Carbohydrates are actively synthesized in the intestine. These synthesized carbohydrates released into the haemolymph could be selectively picked up by the tissues like silkgland, muscle and CNS. Further, the reports of Horie and Tanaka (1957) established that the carbohydrate metabolites generated in intestine get transferred into haemolymph through its wall.

Carbohydrates widely occur in plants in which their quality and quantity vary enormously. Larvae of the 5th instar consume enormous quantity of leaf and perhaps this is the reason for the highest concentrations of carbohydrates in the haemolymph on the 7th day of the 5th instar. The level of reducing and total sugars increases in haemolymph as the larval development progresses, because the unused sugars which are present in the digestive tract appear in haemolymph by passive diffusion [Maurizio 1965].

The decrease on the 8th day of the 5th instar and 1st day of pupa is attributable to the cessation of feeding. The first sign of readiness to moult or spin is usually cessation of feeding by the mature larva. This is followed by a prepupal period during which the gut is emptied and in many lepidopterans the spinning of the cocoon is a major activity prior to pupation.

In silkgland, the levels of total carbohydrates increased from the 1st 2day to the 7th day of the 5th instar, and thereafter decreased on the 8th day of the 5th instar and 1st day of the pupa. Silkgland is a very important organ in the silkworm. It grows enormously during the 5th instar development [Sakaguchi 1978]. The silkgland of *Bombyx mori* becomes very active physiologically and begins to grow rapidly from the middle of the 5th instar. It constitutes roughly 40% of the body weight of the larvae [Ono 1951]. Growth of the silkgland will be the manifestation of accumulation of organic components with particular reference to proteins [Ito 1967; Tazima 1978].

As in other tissues, increase in the total carbohydrate content in the silkgland is attributable to enormous consumption of food during the 5th instar. They are also taken up by the silkgland from the haemolymph. Decrease in content on the 8th day of the 5th instar could be due to their mobilization for energy release for the spinning activity, as this activity demands utilization of greater energy [Yamashita and Hasegawa 1974; Kerkut and Gilbert 1985].

In muscle also the levels of carbohydrates increased up to the 7th day of the 5th instar and decreased on the 8th day of the 5th instar and also on the 1st day of the pupa. Of the several classes of food stuffs available to animals as sources of energy, carbohydrates seem to be of greater significance in muscular contraction. Maheswaramma (1994) estimated the total carbohydrate level in the 5th instar larvae of *Bombyx mori* in different seasons. Siva Prasad (1987) observed that the 5th instar larval development in *Bombyx mori* is accompanied by greater development of body musculature

vis-a-vis its innervation by segmental nerves. Greater muscular activity as the instar progresses would be facilitated by an increase in carbohydrate levels, consequent on greater food intake day by day, reaching the peak on the spinning day. Thereafter the halt in food intake and the ensuing quiescent pupal stage would call for a decrease in carbohydrate levels.

In CNS also the levels of total carbohydrates increased up to the 7th day of the 5th instar and thereafter decreased on the 8th day and also 1st day of the pupa. There is no synthesis of carbohydrates in the CNS, the presence and increasing level might be due to the absorption from the haemolymph. The decreasing levels indicate their mobilization on higher energy demand which could be higher than their accumulation. This mobilization is necessary since the CNS controls the overall activity of the animal, particularly in the spinning period. In agreement with this, higher level of spontaneous electrical activity in the CNS has been observed in the present study at the spinning phase (See Chapter - III).

Comprehensive reviews of carbohydrate metabolism in insects are given by Babers (1941), Yeager and Munson (1941) and Rockstein (1950). Insects derive their energy requirements from the oxidation of carbohydrates [Beenakkers 1969; Sacktor 1975; Weeda and De Kort 1979]. The significance of carbohydrate metabolism has been extensively examined in several insects [Ito and Horie 1959; Stevensen and Wyatt 1964; Brandt and Hüber 1979; Venkatarami Reddy and Benchamin 1990; Sinha *et al.* 1991].

Glycogen

In insects, glycogen is a major nutrient reserve, being stored in the fat body in large amounts, and it is accumulated and utilized in patterns that indicate regulation corresponding to the needs of growth and activity [Wigglesworth 1949; Bade 1962]. The stored glycogen varies greatly in amount and can be mobilized into the haemolymph chiefly as glucose, trehalose or glycerol. Insects, like mammals can synthesize glycogen from uridine disphosphoglucose [Vardanis 1963].

Glycogen is synthesized during periods of active feeding and depleted at times of reduced or no feeding, as during a moult, over the pupal period or during a period of diapause. Its synthesis may be directly from glucose, but it can be derived from amino acids as well. In *Bombyx* [Kuwana 1937] and in *Prodenia* [Babers 1941; Yeager and Munson 1941] blood glucose increases during digestion of food and this is followed by an increase in tissue glycogen. This glycogen decreases throughout the pupal life [Kotake and Sera 1909; Ludwig and Rothstein 1949].

The present study revealed that the level of glycogen in all the tissues increases from the 1st day to the 6th day of the 5th instar and thereafter declines on the 7th day and also on the 1st day of the pupa. Like other animal tissues, insect tissues stored carbohydrates in the form of glycogen for utilization during metamorphosis as an energy source. Besides its role in energy production [Ludwig *et al.* 1965] glycogen is thought to be converted to other sugars that are used for synthetic purposes such as ribose for RNA production [Sasse 1968], deoxyribose, chitin and acid mucopolysaccharides [Van Der Starre - Ven Der Molen 1972].

Since glycogen synthesis and its accumulation occurred mainly during the non-essential feeding period, glycogen storage may not be an essential event for pupal metamorphosis. Thus much of glycogen serves as an energy source during the post-feeding larval period, and the rest is preserved to be utilized by pupae and adults. Therefore, glycogen serves as a predominant reserve store in the tissues during the last larval period. Glycogen is accumulated in *Calliphora* during larval development, most of which is utilized during metamorphosis [Hoglund 1976]. This lends support to the results of the present investigation. The silkworm may more than double its glycogen reserve in 4 days at the end of the larval life apparently by transformation of its protein or fat [Bataillon 1893].

It can be concluded that the sharp decrease in glycogen on the 7th day of the 5th instar and 1st day of the pupa is caused by the cessation of normal feeding. The insect has to necessarily use the stored reserves to satisfy the demands of normal metabolism and of chitin synthesis. In *Philosamia ricini*, the glycogen content falls rapidly during the 1st day of spinning but remains practically constant during subsequent larval-pupal transformation [Bade and Wyatt 1962].

The declining levels of tissue glycogen during the non-feeding period in silkworm is indicative of increased glycogenolysis. Similarly, their increase from the 1st day to the 6th day of the 5th instar is reflective of elevation of glycogenesis and a decline in glycogenolysis in all the tissues. The carbohydrates accumulated through glycogenesis could be utilized through glycogenolysis. During the metamorphosis of many insects as much as 73 to

100% of the stored glycogen may be utilized [Rockstein 1950], a process sometimes correlated with the corresponding increase in fat [Yeager and Munson 1941].

Among the tissues, the muscle seems to possess a high rate of glycogenesis which is followed by the silkgland, CNS and haemolymph in a decreasing order.

Glycogen is known to play a major role in insect muscle contraction [Sacktor 1965] as evidenced by the presence of phosphorylase activity in the tissues of silkworm [Ito and Horie 1959; Shigematsu 1956]. Chadwick and Gilmour [1940] suggested that the energy for insect flight was supplied by the oxidation of carbohydrates stored chiefly as glycogen. This concept was supported by the important study by Williams *et al.* [1943].

It is known that the removal of neurosecretory cells in *Calliphora* [Thomsen 1954] or the corpora allata from *Dixippus* [L'Helias 1953] caused the accumulation of glycogen. The removal of brain from pupae of the silkworm *Bombyx mori* prevented the fall in glycogen associated with pupal development [Kobayashi 1957]. This indicates a role for neural or neurohormonal messages in glycogen metabolism. It has been suggested that the onset of spinning activity is mediated by hormonal messages in silkworm. Consequent on this the vigorous spinning activity without any intake of food could give rise to depletion of glycogen for energy needs.

The blood of *Bombyx mori* contains large amounts of anthrone-reactive material which was neither a reducing sugar sucrose, nor glycogen [Wyatt *et al.* 1956]. Yeager and Munson (1941) demonstrated that during the larval growth of the southern armyworm *Prodenia cridania*, there was a large accumulation of glycogen in the haemocytes which disappeared at the end of the pre-pupal stage. Thus, although to a limited measure, haemolymph may play a role in transporting glycogen to the needy tissues during the active spinning phase.

Thus, accumulation and release of energy from carbohydrates are influenced by feeding or non-feeding stages of silkworms [Pant and Suman Kumar 1979]. However, depending upon the need of the animal, there can be metabolic shifts from lipogenesis to glycogenesis during 5th instar larval development of silkworm under depletion of storage reserves [Inagaki and Yamashita 1986]. Blood sugar and body glycogen are of particular interest in connection with energy stores, carbohydrate utilization, fat-protein-carbohydrate interconversion etc.

The present study indicates that during the 5th instar development the silkworm actively builds up a glycogen reserve through abundant intake of food from the 1st day to the 6th day, and then utilizes the stores for the following non-feeding active spinning phase, for the inactive pupal stage, and again during the non-feeding adult stage.

Glycogen Phosphorylase

Phosphorylase activity may be measured in the direction of glycogen synthesis or breakdown. The effect of glycogen structure on the rate of the phosphorylase reaction has been examined by Childress *et al.* [1970]. In a number of insects glycogen phosphorylase has been shown to exist in two interconvertible forms, viz. phosphorylase 'a' (active) and phosphorylase 'b' (inactive) [Steele 1982]. Isolation and partial characterization of phosphorylase 'a 'and 'b' and partial characterization of phosphorylase 'a' and 'b' have been reported only for the enzyme from flight muscles of *Phormia regina* [Childress and Sacktor 1970] and larval fat body of *Bombyx mori* [Yanagawa and Horie 1978].

In the present study, both active and inactive forms of phosphorylase have been detected in tissues like haemolymph, silkgland, muscle and CNS, during the 5th instar and also on the 1st day of the pupa. The trends of change in these two enzyme activities contrasted each other. The phosphorylase 'a' activity is higher at the beginning of the 5th instar and then gradually decreased at the middle of the 5th instar and thereafter it showed elevated level towards the end of the 5th instar and also on the 1st day of the pupa.

An increase in the activity of phosphorylase 'a' may indicate its active involvement in glycogen degradation or increased glycogen metabolism within the tissues, because glycogen to a varying extent is used as a source of energy in all the tissues. In muscle most or all of the glycogen may be devoted to this function. As the activity of phosphorylase increases the concentration of

glycogen declines. The increase in phosphorylase 'a' activity just prior to pupation could be to fulfill the ensuing need for energy and glucose in chitin synthesis at the time of moulting. This is in consonance with the reports of Yanagava and Horie [1977], who demonstrated that during the 5th larval instar in *Bombyx mori*, phosphorylase activity in midgut, muscle and fatbody is similar as the activity is high at the beginning of the 5th instar, minimal on the 4th day and rises towards the time of moulting. While midgut has equal levels of phosphorylase 'a' and 'b' activities throughout the instar, muscle has considerably greater phosphorylase 'a' activity at all times. It was proposed by Guillory and Mommaerts [1962] that temperature has a role in the rise of phosphorylase 'a' activity.

In the present study, the elevation of phosphorylase 'a' activity is more pronounced in muscle followed by silkgland, haemolymph and CNS. This clearly indicates that in the muscle the glycogen mobilization is greatly favoured over the other tissues.

The activity levels of phosphorylase 'a' during the middle of the 5th instar were found to decrease in the present study suggesting lowered mobilization of glycogen as a source of energy. In *Glossina morsitans* energy is provided mainly by the oxidation of proline. It also signifies the possible retardation exerted on glycogen breakdown at the level of phosphorylase. The observed decrease in the enzyme activity might also be due to other factors. One of them might be the alterations in the protein constituency. Padmavathy *et al.* [1975] reported inhibition of phosphorylase 'a' activity in the presence of increased proteins. Varalakshmi [1998] observed that total

and soluble proteins increased during the 5th instar of silkworm *Bombyx mori*. Another factor might be the possible role of divalant cations like Ca^{++} and Mg^{++} which are known to activate phosphorylase 'b' kinase with simultaneous inhibition of phosphorylase phosphatase [Morgan and Parmeygiani 1963]. Conversion of phosphorylase 'b' to 'a' in homogenates of *Bombyx mori* larval fatbody requires ATP and Mg^{++} [Yanagawa and Horie 1978]. However, the interplay of other factors as above in the 5th instar of *Bombyx mori* in the context of changes in the levels of phosphorylase activity is only conjectural, and requires experimental consolidation.

The active form of phosphorylase can be converted into inactive form by phosphorylase phosphatase, while the conversion of inactive form to active form will be achieved by phosphorylase 'b' kinase. Thus, the phosphorylase kinase and phosphorylase phosphatase were found to be responsible for regulatory behaviour of phosphorylase [Harper 1976].

In contrast to phosphorylase 'a', the activity levels of phosphorylase 'b' were low at the beginning of the 5th instar and thereafter increased and again decreased towards the end of the 5th instar and also on the 1st day of the pupa. It indicates that during early and late stages of the 5th instar and also 1st day of the pupa, phosphorylase 'b' is probably converted into 'a' form. In intact silkmoth pupae, conversion of phosphorylase 'b' to phosphorylase 'a' accompanies stimulated glycogenolysis after wounding or chilling or during pharate adult development [Stevenson and Wyatt 1964; Wiens and Gilbert 1967; Ziegler and Wyatt 1975]. Contraction of muscle is accompanied by conversion of phosphorylase 'b' to 'a' [Childress and Sacktor 1970;

Achazi *et al.* 1975; Kallapur and Narasubhu 1976]. In the event of decrease of phosphorylase 'a', the inactive form probably compensates by conversion into the active form. Existence of such a situation for the increasing levels of phosphorylase 'b' during the middle of the 5th instar requires examination.

Glycogen could be the major fuel utilized during the transition periods in silkworm. One would expect to find that the potential phosphorylase activity matched the estimated rate of carbohydrate consumption. Childress and Sacktor [1970] estimated that a 70% level of phosphorylase 'a' can account for the release of 4.3 μ moles of glycosyl residues from glycogen. Thus phosphorylase activity may play a key role in regulating the availability of glucosyl units, either as a source of energy or as "building blocks" for biosynthetic reactions during the 5th instar development of *Bombyx mori*.

Glucose

In insects glucose is a major nutrient and serves as an energy source and a multi-functional precursor of the synthesis of trehalose, glycogen, lipids, amino acids and proteins [Chippendale 1978]. Therefore glucose seems to be a representative substrate for studying the nutrients ingested by the insect. Trehalose in the intestine is split into glucose which is easily absorbed into the haemolymph [Wyatt 1967]. Glucose absorption is generally a passive process in insects [Turumen 1983].

The present study showed increased glucose levels from the 1st day to 7th day of the 5th instar and declined on the 1st day of the pupa. However,

the change from the 7th day of the 5th instar to the 1st day of the pupa was little. Higher levels of glucose were detected in the haemolymph, followed by silkgland, muscle and CNS. Haemolymph is the chief transport medium for metabolites. It has been established in insects that high levels of blood glucose are necessary due to the relative inefficiency of the open circulatory system and the need to meet the required energy during flight and fight situations [Keeley 1978].

It is highly probable that in silkworms the haemolymph glucose is partly derived from glycogen and partly from the hydrolysis of trehalose. A similar situation exists in honeybee [Brandt and Hüber 1979]. The elevation in the levels of haemolymph glucose recorded in the present investigation is once again reflective of the higher levels in the tissues since the haemolymph has no synthetic machinery of its own. Certain other factors like stress and exercise might also cause a rise in the levels of haemolymph glucose during summer as proposed by Willson and Rounds [1972] and Matthews *et al.* [1976a]. These factors, however, are not considered in the present study. In this regard a mention may be made of the role of insect haemolymph sugar which is known to undergo hydrolysis into glucose, part of which is incorporated into the muscle [Ford and Candy 1972; Murphy and Wyatt 1975; Brandt and Hüber 1979]. In *Bombyx mori*, a number of carbohydrates including glucose, fructose, sucrose, maltose and raffinose were more effective than others in increasing trehalose in the haemolymph [Horie 1961].

In the tissues like muscle, silkgland and CNS, increasing glucose levels indicate its increased uptake into the tissues from the breakdown of the food

taken in or its decreased mobilization into the glycolytic cycle. The elevation in glucose level could also be attributed to its reduced oxidation in glycolysis [Edwards 1973]. It is also possible that the glucose derived from the enormous consumption of food is probably quite in excess of what is utilized, consequently resulting in an increased level of glucose as the instar progressed.

On commencement of spinning a significant fall in glucose is observed, suggesting that glucose is utilized as a source of energy for spinning. Glucose increases during development of the blowfly *Calliphora erythrocephala* and reaches a peak of 1.2 mg/g wet weight until puparium formation [Hanne Duve 1976].

Although glucose in the tissues is not necessary for the feeding period of 5th instar, its presence and increasing levels are explained by the fact that the rate of phosphorylation of glucose might be slower than the diffusion rate of glucose from the intestine or fat body. In insects, however, the absorption of glucose from the midgut has been shown to be by passive diffusion coupled to its capture and conversion to trehalose in the fat body [Treherne 1958; Shyamala and Bhat 1965].

Thus the day-to-day changes in glucose levels during the 5th instar in *Bombyx mori* clearly indicate its build-up during the early periods for its utilization for energy for the active spinning phase later.

Trehalose

Trehalose is an important carbohydrate reported to be present in the blood of insects including *Bombyx mori*. Wyatt and Kalf [1957] reported that the major blood carbohydrate in insects is trehalose, a non-reducing disaccharide and that it is possibly an important reserve carbohydrate in insects. Fairbrain [1958] also reported the existence of trehalose in several other invertebrates. It has been demonstrated that trehalose is actively synthesized in the fat bodies of insects and it rapidly decreases during active movement and starvation [Clegg and Evans 1961; Saito 1963]. Recently, Shamitha [1998] estimated trehalose content in *Antheraea mylitta* under outdoor and total indoor conditions.

The present study revealed that the level of trehalose in all the tissues increased from the 1st day to the 7th day of the 5th instar, and thereafter it declined on the 1st day of the pupa. Trehalose is the principal sugar of silkworms. Like in other insects it could get accumulated during the process of growth and development, and reach the maximum level before the spinning stage in silkworm. The observation of Dinesh and Purushotham Rao (1993) supports this assumption. In all the tissues the day-to-day percent increase in trehalose content during the 5th instar was enormous.

In *Bombyx mori*, the total body weight rises steadily during the larval life, particularly during the 5th instar until a day before they start spinning, and then falls precipitously during the spinning period to about half its maximum value [Heller and Sweichowska 1948]. The 5th instar alone covers

about 87.67% of the total food consumed for the entire larval life [Matsumura and Takeuchi 1950]. Further, the length of the 5th instar larval life is more than twice that of each of the preceeding instars and the quantity of food consumed increases day by day, the highest being on the day before spinning [Horie and Watanabe 1983]. However, the very high content of trehalose is not universal in insects, and in the adult honeybee the amount of free reducing sugar is only 1.4% in the haemolymph [Wyatt 1961].

The decrease on the 1st day of pupa in all tissues of the silkworm might be due to the ready availability of trehalose as an immediate carbohydrate reserve, which is utilized to meet the energy needs.

Of the tissues examined, haemolymph showed the highest trehalose content, followed by the silkgland, muscle and CNS. The volume of body fluid of the silkworm increases by about 4 times during the 5th instar [Nittono 1961]. The major source of trehalose in the haemolymph appears to be from the breakdown of glycogen in the fat body [Steele 1963; Matthews and Downer 1974]. Wyatt and Kalf [1956] reported the discovery of large amounts of the non-reducing disaccharide trehalose in the haemolymph of the larvae and pupae of several lepidopteran insects. The haemolymph trehalose levels respond strikingly to the nutritional state, quality and quantity of food intake [Hansen 1964], to the developmental stages of insects [Howden and Kilby 1960], and to the physiological condition [Nowosielski and Patton 1964]. It was reported that trehalose diffuses from the haemolymph according to the concentration into the gut and then degrades into glucose to meet the energy needs [Jabbar and Mahamed 1990]. In some insect species the fall in blood

trehalose level during muscular activity or starvation is due to the fact that trehalose acts as an immediately available carbohydrate reserve [Saito 1960; Clegg and Evans 1961]. Therefore blood glucose is maintained at a relatively stable level by a dynamic equilibrium between the synthesis and breakdown of trehalose.

Trehalose levels in haemolymph are closely related to moulting, metamorphosis and diapause [Wyatt 1967]. In *Hyalophora cecropia* [Jungreis and Wyatt 1972] and in *Bombyx mori* [Hirano and Yamashita 1980], trehalose concentration in haemolymph declined to half or less at the transformation of larva to pupa. This decline in trehalose was interpreted as due to a decrease in its synthesis and not to increased utilization by other tissues [Hirano and Yamashita 1980]. Evidently, trehalose plays a relatively minor role as an energy reserve during metamorphosis. It may be more significant in carbohydrate transport, perhaps conveying glucose units from fatbody glycogen to the sites of metabolism in other tissues. The synthesis of haemolymph trehalose seems to be controlled by numerous factors including supply of precursors, enzyme activity and concentration of regulators [Jungreis and Wyatt 1972].

Trehalose is quantitatively more important than glycogen as the form in which carbohydrate is stored in the silkglands of *Bombyx mori*. The accumulation of trehalose proceeds along with that of the silk protein in the silkgland during the 5th instar [Shigematsu and Takeshita 1962] and attains the maximum immediately before spinning the cocoon. Thereafter the level of trehalose decreases along with the use of the silkglands which shrink owing to the spinning of the cocoon and histolysis which occurs during larval-

pupal transformation. These observations lend support to the present investigation. These results would suggest that the accumulation of trehalose during the 5th instar is necessary for either growth or maintainance of the gland and in the later part used for the formation of cocoon or through conversion into protein, or for other metabolic purposes.

In muscle also the accumulation of trehalose increased during the 5th instar and declined on the 1st day of the pupa. The increase is attributable to the increased food consumption. The high level of trehalose even after the stopping of food intake during the spinning phase could be due to rapid breakdown of the stored carbohydrates and their conversion into trehalose for energy for active spinning. The fall on the 1st day of the pupa is indicative of the reduced energy requirements and the mobilization of trehalose into the energy stores for utilization during the pupal and adult life.

A decrease in trehalose synthesis during metamorphosis coincides with an increase in glycogen synthesis in fat body [Shigematsu 1956; Yamashita and Hasegawa 1974] suggesting a shift from the intermediary metabolism to storage in anticipation of the adult development. Such a change in *Bombyx mori* fat body has been shown for storage proteins which are synthesized and released into haemolymph during feeding but are sequestered by the fat body prior to adult development [Tojo *et al.* 1981]. Therefore, the metabolic shift in fatbody directed to metamorphosis is effectively reflected by the changes in haemolymph trehalose levels of *Bombyx mori*.

The present study demonstrates trehalose as one of the primary sources of energy for growth and development of the silkworm, especially during the 5th instar. The tremendous change this sugar undergoes day to day during this instar is ample proof of its importance in energy release, while also indicating its possible involvement in metabolic interconversions towards energy storage for utilization during the pupal stage and adult life.

Trehalase

Trehalase, the enzyme that breaks down trehalose, is responsible for trehalose metabolism in insect tissues [Wyatt 1960]. High trehalase activity in insects has been found particularly in the intestinal epithelium and digestive system [Saito 1960; Gilby *et al.* 1967; Pant and Morris 1974; Yamashita *et al.* 1974]. Trehalase plays a significant role in the supply of energy in the insects [Wyatt 1967] and the activity of trehalase might serve as an indicator of energy reserves resulting from availability of carbohydrate nutrients.

The present study shows that the activity levels of trehalase are higher in the early and late stages of the 5th instar and also on the 1st day of the pupa. The middle of the 5th instar showed lower activity levels.

The activity levels of trehalase during the early and later stages of the 5th instar show that the enzyme activity is relatively very high throughout the 5th instar, which suggests high metabolism of carbohydrates by the silkworm. An increase in the activity of this enzyme indicates increased

trehalose metabolism in the tissues. High activity of trehalase during the moult and early stages of Eri silkworm were reported by Chang *et al.* [1964].

The increasing trehalase activity during the 5th instar together with increasing amounts of trehalose and a minor increase in glucose content can be explained by the fact that for the preparation for spinning activity and for puparium formation a lot of energy is needed.

In 1961, Steele reported that the corpora cardiaca extract increases trehalase level in the haemolymph of the American cockroach. Recently, a neurohormone called hypertrehalosamine has been isolated from the whole head of the adult cockroach *Blaberus discoidalis* which mobilizes trehalose from the fatbody glycogen store. In the light of this, high activity of trehalase during the spinning period may perhaps be presumed as due to the activation of this enzyme by neurosecretory activity of the brain. This assumption, however, requires experimental support.

Of the tissues examined, silkgland showed higher trehalase activity followed by the muscle, CNS and haemolymph. Shimada [1975] demonstrated that the trehalase activity in silkgland cells is low through much of the 5th instar, but increases greatly during the spinning stage. In the muscle the energy stores may be used for co-ordinated muscular activity especially during cocoon spinning. Trehalase is widely distributed in insect tissues such as muscle and gut [Gussin and Wyatt 1965; Gilby *et al.* 1967; Yanagawa 1971]. The metabolic use of trehalose depends on its hydrolysis to glucose in

the presence of trehalase. Trehalase occurs in the muscles and the glucose produced is utilized as a fuel for flight by *Phormia, Locusta* and *Periplaneta* [Wyatt 1967].

In the present study haemolymph showed very low activity of trehalase. It is known that the haemolymph content is less during the early stages of an insect and the volume of haemolymph and haemocytes are high during the late stages of the insect development [Tazima 1978]. The presence of trehalase in the haemolymph and fatbody [Howden and Kilby 1956] and other tissues in insects including the silkworm, *Bombyx mori* and *Phormia ricini* [Pant and Morris 1972; Unni 1991] has been reported. Recenlty, trehalase activity was identified in the haemolymph and silkgland of *Bombyx mori* during 5th instar [Dinesh and Purushotham Rao 1993] and in *Antheraea assama* [Unni *et al.* 1997] during the spinning period.

Higher activity of trehalase during the early and late stages of 5th instar is presumably to meet the energy demands because the enzyme trehalase hydrolyses trehalose to glucose. Higher trehalase activity in the tissues is indicative of higher conversion of glucose during energy need. The decrease in trehalase activity during the middle stage of the 5th instar, while there is a simultaneous increase in trehalose level, is probably indicative of facilitation of trehalose accumulation through a decrease in trehalase activity. This, however, is only a transient phase. Further, the increase in trehalose level could be both due to greater food intake as well as a decrease in trehalase activity, and so trehalose changes cannot be solely attributed to enzyme activity changes alone.

In the Eri silkworm, Chang *et al.* [1964] reported that the trehalase activity in the haemolymph was detected only during fasting and moulting and its inactivity is due to the presence of inhibiting substances in the haemolymph. Magnesium ion is regarded as a trehalase inhibitor [Derr and Randall 1966], but Saito [1960] found that neither Magnesium chloride nor glycogen could inhibit trehalase in the silkworm. It should be admitted that the regulation of trehalase activity is poorly understood. It may be assumed that hormonal control and a glucose feedback mechanism may also be involved in the regulation of trehalase activity.

Thus trehalose and trehalase probably play a very important role in regulating the blood sugar level, glycogen synthesis in fat body, larval-pupal transformation and other physiological and functional properties of the silkgland during the metamorphosis of *Bombyx mori*.

Table 1: Day - to - day changes in the levels of **total carbohydrates** during the 5th instar development of *Bombyx mori*. Each value, expressed as mg of glucose / g wet wt of the tissue or 1 ml of haemolymph, is the mean ± standard deviation (SD) of six observations. For each observation tissue from about 50 animals was pooled. The percent changes for all days were calculated taking the first day of the 5th instar as the control.

Name of the tissue		Days of 5th Instar								First day of pupa
		1	2	3	4	5	6	7	8	
Haemolymph	Mean	6.27	8.17	10.95	13.46	16.27	19.28	22.47	14.40	10.55
	SD	± 0.51	± 0.51	± 0.39	± 1.10	± 0.64	± 0.46	± 0.51	± 0.38	± 0.80
	% Change		+ 30.34	+ 74.68	+ 114.84	+ 159.54	+ 207.68	+ 258.59	+ 129.76	+ 68.35
Silkgland	Mean	3.23	4.72	7.25	9.08	12.30	15.35	17.73	10.14	8.15
	SD	± 0.13	± 0.19	± 0.21	± 0.50	± 0.82	± 0.10	± 0.19	± 0.19	± 0.76
	% Change		+ 46.13	+ 124.09	+ 181.21	+ 280.70	+ 370.23	+ 448.96	+ 213.77	+ 152.32
Muscle	Mean	2.31	3.37	4.44	6.05	8.43	10.57	13.88	7.32	4.15
	SD	± 0.23	± 0.18	± 0.13	± 0.24	± 0.32	± 0.30	± 0.20	± 0.26	± 0.32
	% Change		+ 46.62	+ 93.18	+ 163.16	+ 266.71	+ 359.89	+ 504.00	+ 218.41	+ 80.92
CNS	Mean	1.01	1.58	2.55	3.33	5.24	6.21	8.43	5.13	2.06
	SD	± 0.11	± 0.13	± 0.110	± 0.12	± 1.13	± 0.25	± 0.33	± 0.15	± 0.25
	% Change		+ 56.43	+ 152.97	+ 229.70	+ 418.81	+ 514.35	+ 734.48	+ 408.25	+ 103.96

All changes are statistically significant at $P < 0.001$.

LEGEND FOR FIGS. 1 TO 4

Histograms showing day-to-day per cent changes in the levels of total carbohydrates in the haemolymph (Fig.1), silkgland (Fig.2), muscle (Fig.3) and CNS (Fig.4) from the 2nd day to the 7th day of the 5th instar and also on the 1st day of the pupa in *Bombyx mori*. The level on the 1st day was taken as the control (C) for calculating the change on the remaining days.

Fig.1

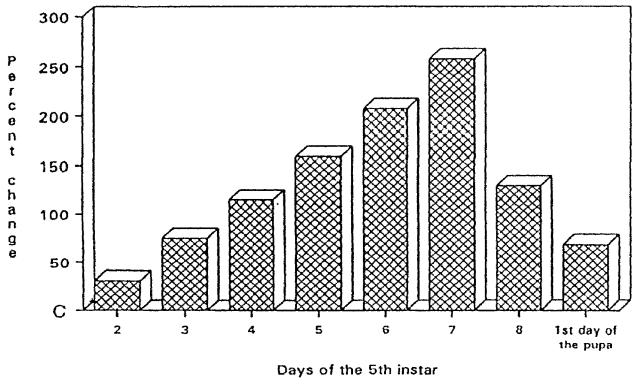


Fig.2

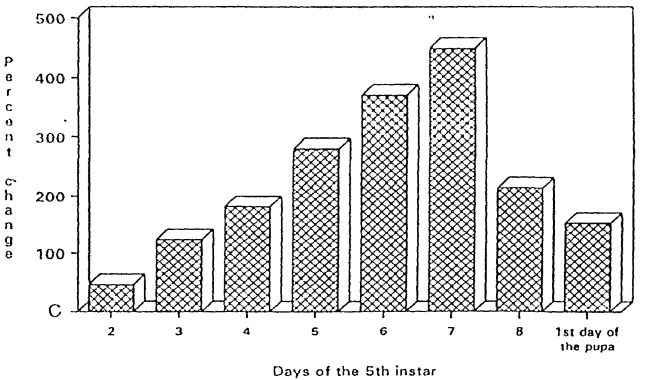


Fig.3

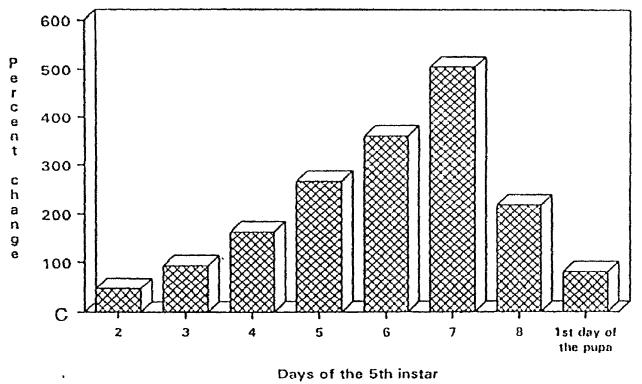


Fig.4

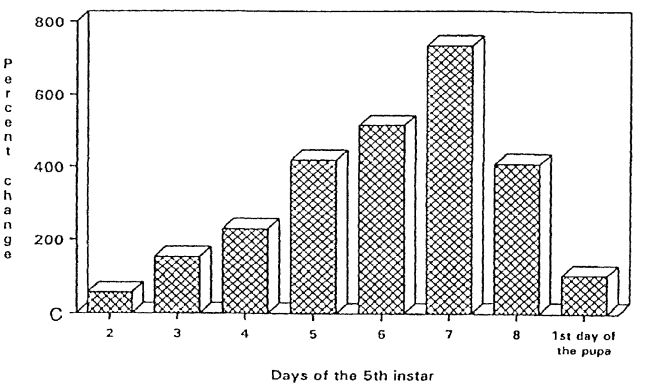


Table 2: Day - to - day changes in the levels of **glycogen** during the 5th instar development of *Bombyx mori*. Each value, expressed as mg of glucose / g wet wt of the tissue or 1 ml of haemolymph, is the mean ± standard deviation (SD) of six observations. For each observation tissue from about 50 animals was pooled. The percent changes for all days were calculated taking the first day of the 5th instar as the control.

Name of the tissue		Days of 5th Instar							First day of pupa
		1	2	3	4	5	6	7	
Haemolymph	Mean	1.42	1.84	2.44	2.76	2.95	3.42	1.57	0.75
	SD	± 0.06	± 0.15	± 0.12	± 0.16	± 0.24	± 0.31	± 0.14	± 0.31
	% Change		+ 30.17***	+ 72.18***	+ 94.82***	+ 108.19***	+ 141.21***	+ 11.05*	- 47.24***
Silkgland	Mean	2.61	3.16	3.70	4.42	6.63	7.09	3.90	1.14
	SD	± 0.95	± 1.20	± 0.35	± 0.32	± 0.11	± 0.20	± 0.62	± 0.38
	% Change		+ 21.08NS	+ 41.74*	+ 69.59**	+ 154.29***	+ 171.70***	+ 49.30*	- 56.10**
Muscle	Mean	2.88	4.12	6.02	6.94	8.37	11.02	3.86	0.50
	SD	± 0.57	± 0.36	± 0.66	± 0.25	± 0.65	± 0.49	± 1.19	± 0.52
	% Change		+ 43.03**	+ 108.98***	+ 140.85***	+ 190.45***	+ 282.40***	+ 33.86NS	- 82.99***
CNS	Mean	0.17	0.41	0.66	1.04	1.58	1.80	0.36	0.08
	SD	± 0.03	± 0.09	± 0.12	± 0.06	± 0.17	± 0.19	± 0.07	± 0.04
	% Change		+ 138.24***	+ 279.96***	+ 501.05***	+ 813.77***	+ 938.72***	+ 105.39NS	- 53.75NS

*** = $P < 0.001$; ** = $P < 0.01$; * = $P < 0.05$; NS : Not significant

LEGEND FOR FIGS. 5 TO 8

Histograms showing day-to-day per cent changes in the levels of **glycogen** in the haemolymph (Fig.5), silkgland (Fig.6), muscle (Fig.7) and CNS (Fig.8) from the 2nd day to the 7th day of the 5th instar and also on the 1st day of the pupa in *Bombyx mori*. The level on the 1st day was taken as the control (C) for calculating the change on the remaining days.

Fig.5

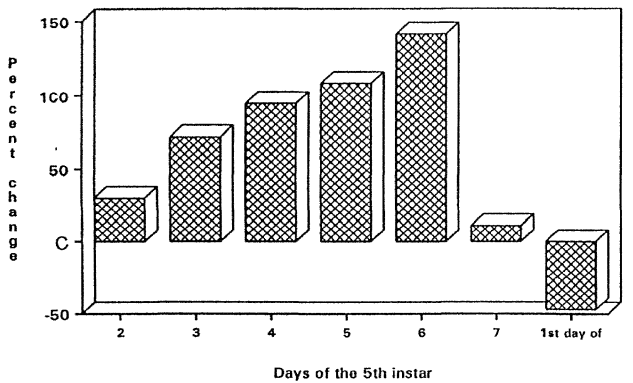


Fig.6

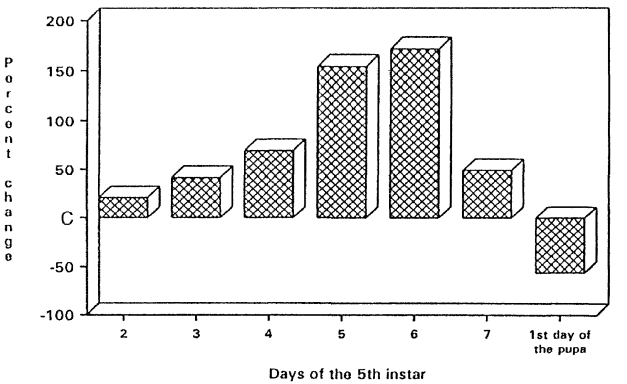


Fig.7

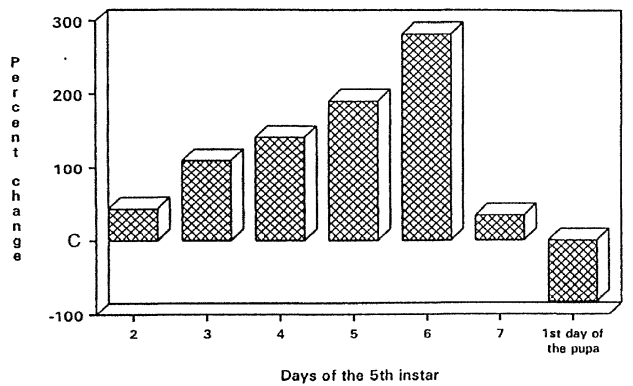


Fig.8

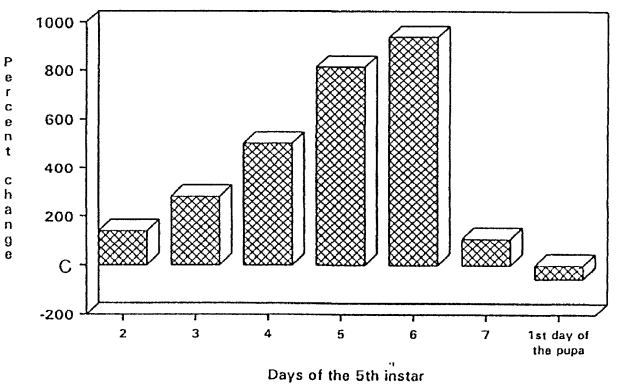


Table 3: Day - to - day changes in the levels of **glycogen phosphorylase 'a'** during the 5th instar development of *Bombyx mori*. Each value, expressed as mg of glucose / g wet wt of the tissue or 1 ml of haemolymph, is the mean ± standard deviation (SD) of six observations. For each observation tissue from about 50 animals was pooled. The percent changes for all days were calculated taking the first day of the 5th instar as the control.

Name of the tissue		Days of 5th Instar							First day of pupa
		1	2	3	4	5	6	7	
Haemolymph	Mean	6.05	4.79	4.03	3.44	3.33	3.97	4.35	5.41
	SD	± 0.21	± 0.23	± 0.21	± 0.41	± 0.24	± 0.24	± 0.38	± 0.36
	% Change		- 20.73***	- 33.38***	- 43.17***	- 44.94***	- 34.35***	- 28.09***	- 10.58**
Silkgland	Mean	6.83	6.60	5.16	4.97	4.34	3.91	5.17	6.69
	SD	± 0.32	± 0.36	± 0.42	± 0.21	± 0.39	± 0.23	± 0.36	± 0.45
	% Change		- 3.46NS	- 24.42***	- 27.32**	- 36.49***	- 42.83***	- 24.39*	- 2.07NS
Muscle	Mean	8.24	7.88	6.79	5.92	5.13	4.68	6.12	8.01
	SD	± 0.64	± 0.64	± 0.51	± 0.38	± 0.29	± 0.50	± 0.27	± 0.46
	% Change		- 4.39NS	- 17.60**	- 28.10***	- 37.79***	- 43.17***	- 25.69***	- 2.73NS
CNS	Mean	4.47	3.33	2.79	2.22	2.05	2.60	4.80	4.88
	SD	± 0.45	± 0.43	± 0.32	± 0.58	± 0.26	± 0.41	± 0.28	± 0.45
	% Change		- 25.49***	- 37.72***	- 50.42***	- 54.15***	- 41.89***	+ 7.34NS	+ 9.13NS

*** = P < 0.001; ** = P < 0.01; * = P < 0.05; NS : Not significant.

LEGEND FOR FIGS. 9 TO 12

Histograms showing day-to-day per cent changes in the levels of **glycogen phosphorylase 'a'** in the haemolymph (Fig.9), silkgland (Fig.10), muscle (Fig.11) and CNS (Fig.12) from the 2nd day to the 7th day of the 5th nstar and also on the 1st day of the pupa in *Bombyx mori*. The level on the .st day was taken as the control (C) for calculating the change on the emaining days.

Fig.9

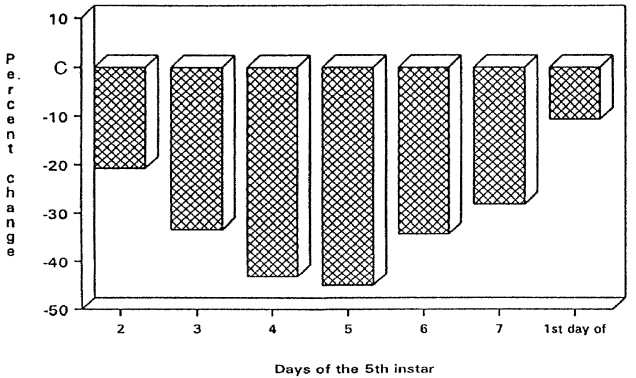


Fig.10

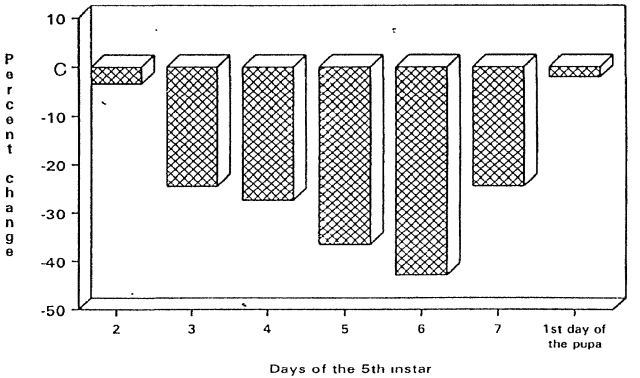


Fig.11

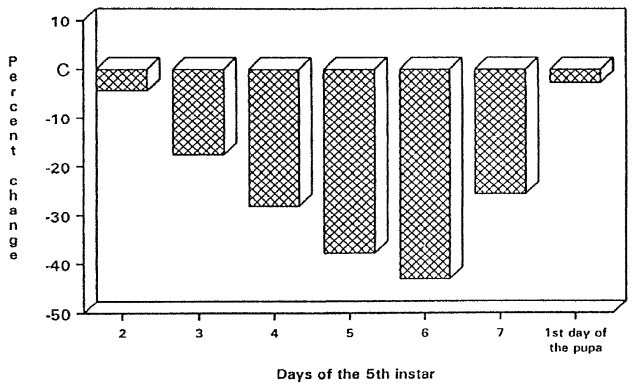


Fig.12

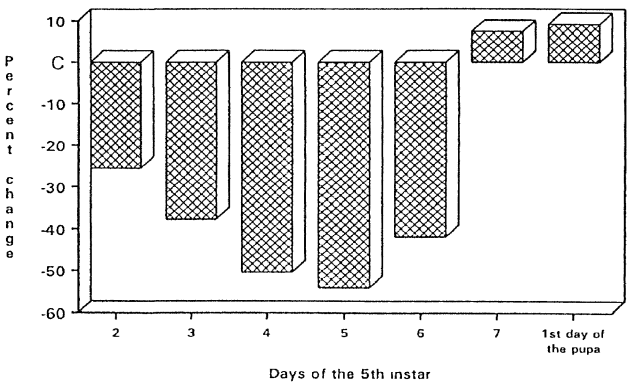


Table 4: Day - to - day changes in the levels of **glycogen phosphorylase 'b'** during the 5th instar development of *Bombyx mori*. Each value, expressed as mg of glucose / g wet wt of the tissue or 1 ml of haemolymph, is the mean ± standard deviation (SD) of six observations. For each observation tissue from about 50 animals was pooled. The percent changes for all days were calculated taking the first day of the 5th instar as the control.

Name of the tissue		Days of 5th Instar							First day of pupa
		1	2	3	4	5	6	7	
Haemolymph	Mean	2.94	3.84	4.53	5.07	6.07	5.64	5.93	4.52
	SD	± 0.13	± 0.10	± 0.12	± 0.19	± 0.16	± 2.52	± 0.17	± 0.13
	% Change		+ 30.44***	+ 54.02***	+ 72.50***	+ 106.29***	+ 91.83*	+ 101.81***	+ 53.62**
Silkgland	Mean	2.34	3.19	5.31	5.65	6.38	6.99	4.19	3.80
	SD	± 0.07	± 0.08	± 0.13	± 0.09	± 0.08	± 0.06	± 0.28	± 0.08
	% Change		+ 36.37**	+ 127.31***	+ 141.86***	+ 173.18***	+ 199.21***	+ 79.45*	+ 62.71***
Muscle	Mean	3.38	4.50	5.56	6.08	7.39	8.22	5.31	4.96
	SD	± 0.09	± 0.16	± 0.26	± 0.18	± 0.33	± 0.13	± 0.12	± 0.16
	% Change		+ 33.33***	+ 64.79***	+ 80.04***	+ 177.13***	+ 143.40***	+ 57.23***	+ 46.81***
CNS	Mean	2.40	2.58	3.00	3.50	5.64	6.16	4.01	3.67
	SD	± 0.18	± 0.07	± 0.09	± 0.28	± 0.35	± 0.35	± 0.15	± 0.33
	% Change		+ 7.67NS	+ 25.20***	+ 45.90***	+ 135.51***	+ 156.88***	+ 67.36***	+ 53.00***

*** = $P < 0.001$; ** = $P < 0.01$; * = $P < 0.05$; NS: Not significant.

LEGEND FOR FIGS. 13 TO 16

Histograms showing day-to-day per cent changes in the levels of **glycogen phosphorylase 'b'** in the haemolymph (Fig.13), silkgland (Fig.14), muscle (Fig.15) and CNS (Fig.16) from the 2nd day to the 7th day of the 5th instar and also on the 1st day of the pupa in *Bombyx mori*. The level on the 1st day was taken as the control (C) for calculating the change on the remaining days.

Fig.13

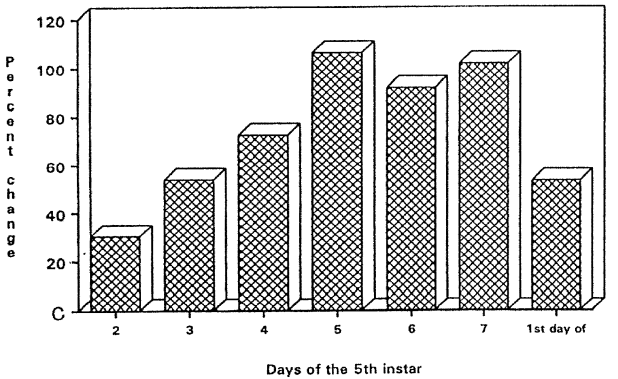


Fig.14

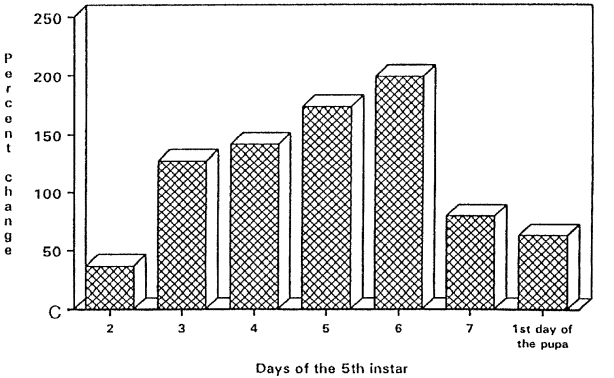


Fig.15

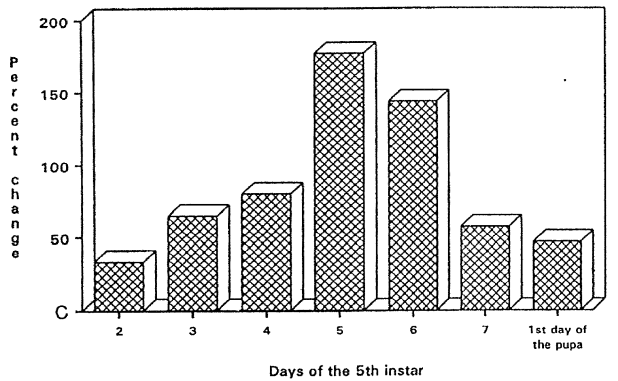


Fig.16

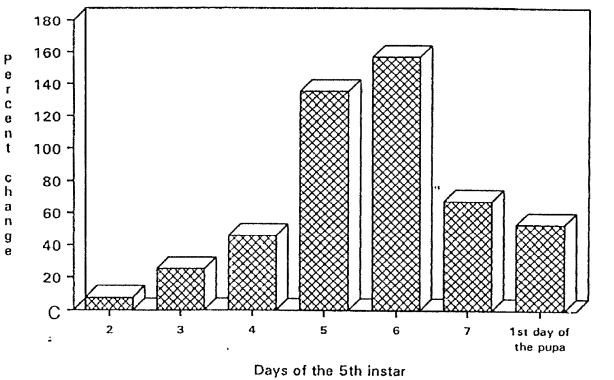


Table 5: Day - to - day changes in the levels of **glucose** during the 5th instar development of *Bombyx mori*. Each value, expressed as mg of glucose / g wet wt of the tissue or 1 ml of haemolymph, is the mean ± standard deviation (SD) of six observations. For each observation tissue from about 50 animals was pooled. The percent changes for all days were calculated taking the first day of the 5th instar as the control.

Name of the tissue		Days of 5th Instar							First day of pupa
		1	2	3	4	5	6	7	
Haemolymph	Mean	1.42	2.53	3.04	3.29	4.85	5.19	7.68	6.62
	SD	± 0.45	± 0.22	± 0.35	± 0.59	± 0.72	± 0.22	± 0.80	± 0.19
	% Change		+ 78.22***	+ 114.44***	+ 131.82***	+ 241.74***	+ 265.41***	+ 441.26***	+ 366.66***
Silkgland	Mean	1.02	1.26	1.68	1.89	2.49	3.21	5.57	4.55
	SD	± 0.10	± 0.11	± 0.19	± 0.17	± 0.60	± 0.29	± 0.60	± 0.37
	% Change		+ 23.14**	+ 64.36***	+ 84.83***	+ 143.66***	+ 214.17***	+ 445.01***	+ 345.24***
Muscle	Mean	1.00	1.39	1.52	1.35	2.00	2.20	3.18	2.73
	SD	± 0.01	± 0.10	± 0.19	± 0.16	± 0.09	± 0.11	± 0.45	± 0.05
	% Change		+ 38.38***	+ 51.87***	+ 34.18***	+ 98.95***	+ 119.82***	+ 216.63***	+ 172.18***
CNS	Mean	0.19	0.29	0.44	0.53	0.68	0.88	0.97	0.10
	SD	± 0.01	± 0.04	± 0.04	± 0.12	± 0.20	± 0.30	± 0.52	± 0.04
	% Change		+ 48.96***	+ 127.83***	+ 172.16***	+ 251.03***	+ 354.12***	+ 402.06**	- 51.03**

*** = $P < 0.001$; ** = $P < 0.01$.

LEGEND FOR FIGS. 17 TO 20

Histograms showing day-to-day per cent changes in the levels of **glucose** in the haemolymph (Fig.17), silkgland (Fig.18), muscle (Fig.19) and CNS (Fig.20) from the 2nd day to the 7th day of the 5th instar and also on the 1st day of the pupa in *Bombyx mori*. The level on the 1st day was taken as the control (C) for calculating the change on the remaining days.

Fig.17

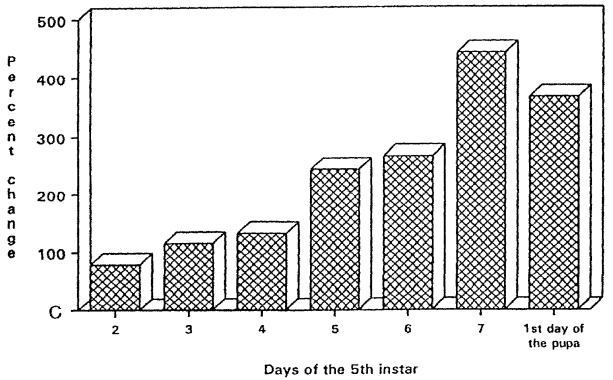


Fig.18

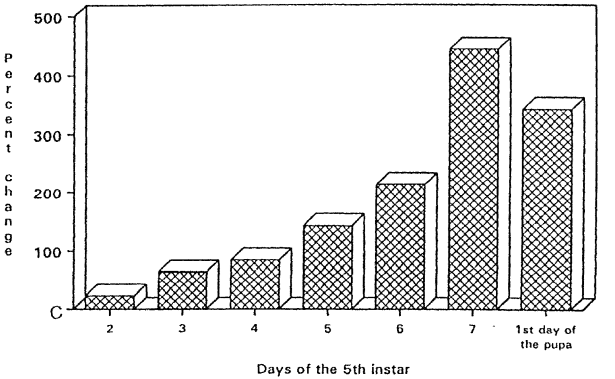


Fig.19

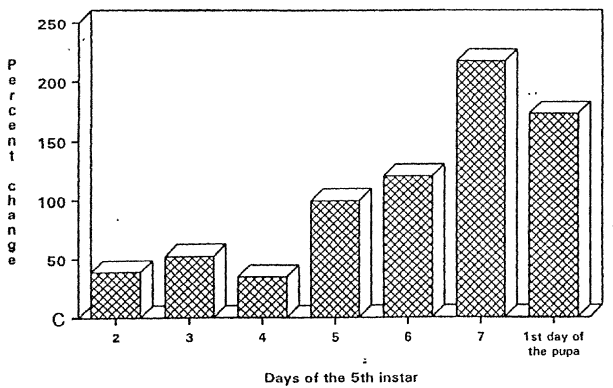


Fig.20

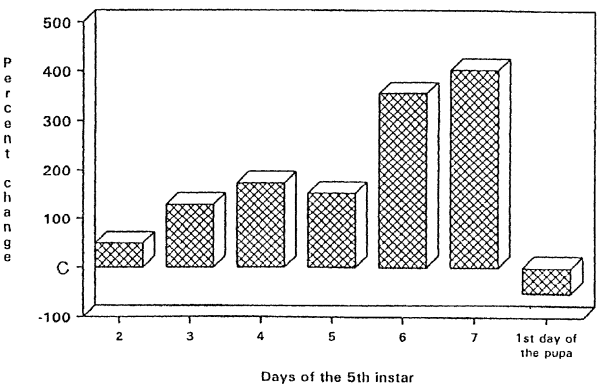


Table 6: Day - to - day changes in the levels of **trehalose** during the 5th instar development of *Bombyx mori*. Each value, expressed as mg of glucose / g wet wt of the tissue or 1 ml of haemolymph, is the mean ± standard deviation (SD) of six observations. For each observation tissue from about 50 animals was pooled. The percent changes for all days were calculated taking the first day of the 5th instar as the control.

Name of the tissue		Days of 5th Instar							First day of pupa
		1	2	3	4	5	6	7	
Haemolymph	Mean	1.00	3.65	6.10	8.29	9.34	10.64	12.17	6.31
	SD	± 0.18	± 0.88	± 0.87	± 0.53	± 0.62	± 1.15	± 1.44	± 0.62
	% Change		+ 269.31	+ 517.65	+ 739.08	+ 845.80	+ 976.45	+ 1131.39	+ 538.36
Silkgland	Mean	0.46	1.58	2.83	4.61	6.25	8.46	9.67	6.17
	SD	± 0.05	± 0.37	± 0.55	± 0.46	± 0.54	± 1.11	± 0.90	± 0.55
	% Change		+ 242.07	+ 514.50	+ 901.69	+ 1256.96	+ 1735.99	+ 1998.82	+ 1239.77
Muscle	Mean	0.24	0.96	1.53	3.33	5.35	7.06	8.36	4.69
	SD	± 0.06	± 0.10	± 0.28	± 0.45	± 0.44	± 0.22	± 0.42	± 0.54
	% Change		+ 307.31	+ 551.53	+ 1316.82	+ 2174.14	2902.55	+ 3454.84	+ 1892.77
CNS	Mean	0.11	0.57	0.86	1.74	3.27	4.93	5.90	3.21
	SD	± 0.02	± 0.05	± 0.06	± 0.16	± 0.27	± 0.23	± 0.17	± 0.27
	% Change		+ 405.71	+ 667.85	+ 1451.25	+ 2819.28	+ 4304.10	+ 5167.85	+ 2769.28

All changes are statistically significant at $P < 0.001$.

LEGEND FOR FIGS. 21 TO 24

Histograms showing day-to-day per cent changes in the levels of **trehalose** in the haemolymph (Fig.21), silkgland (Fig.22), muscle (Fig.23) and CNS (Fig.24) from the 2nd day to the 7th day of the 5th instar and also on the 1st day of the pupa in *Bombyx mori*. The level on the 1st day was taken as the control (C) for calculating the change on the remaining days.

Fig.21

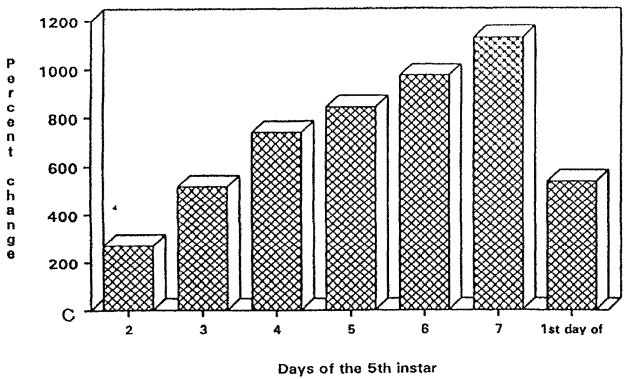


Fig.22

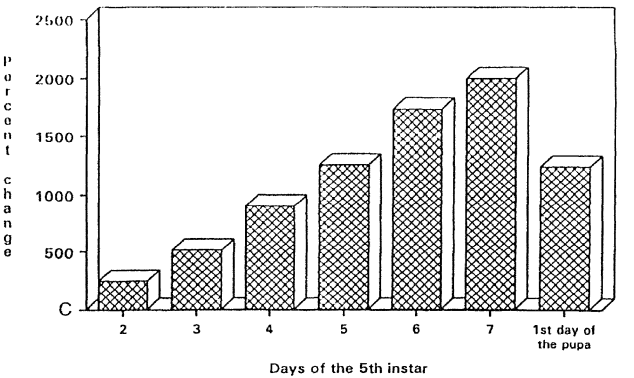


Fig.23

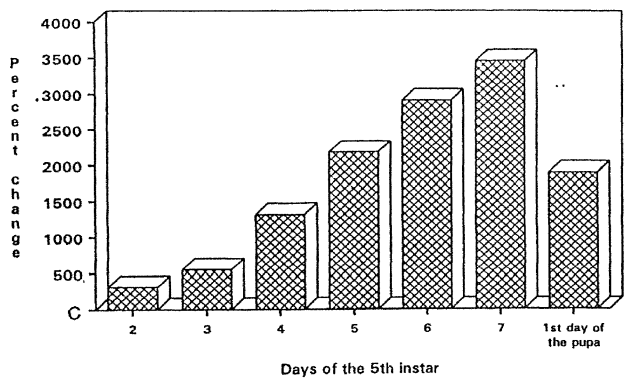


Fig.24

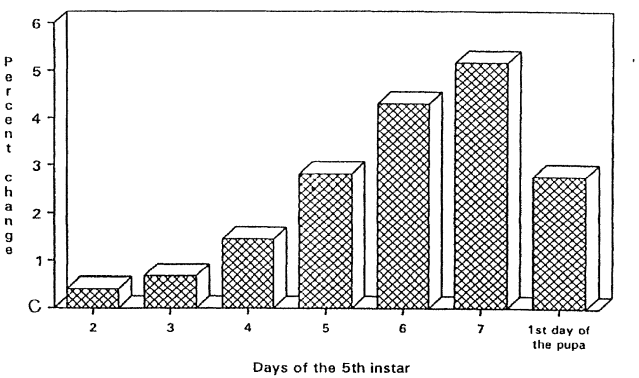


Table 7: Day - to - day changes in the levels of **trehalase** during the 5th instar development of *Bombyx mori*. Each value, expressed as mg of glucose / g wet wt of the tissue or 1 ml of haemolymph, is the mean ± standard deviation (SD) of six observations. For each observation tissue from about 50 animals was pooled. The percent changes for all days were calculated taking the first day of the 5th instar as the control.

Name of the tissue		Days of 5th Instar							First day of pupa
		1	2	3	4	5	6	7	
Haemolymph	Mean	0.098	0.059	0.050	0.020	0.008	0.009	0.013	0.1362
	SD	± 0.007	± 0.008	± 0.006	± 0.002	± 0.003	± 0.002	± 0.004	± 0.019
	% Change		- 39.38***	- 48.45***	- 79.01***	- 91.00***	- 90.66***	- 86.58***	+ 38.74**
Silkgland	Mean	1.198	0.172	0.129	0.042	0.043	0.028	0.008	0.492
	SD	± 0.025	± 0.006	± 0.009	± 0.007	± 0.009	± 0.005	± 0.040	± 0.020
	% Change		- 12.78***	- 34.56***	- 78.72***	- 78.13***	- 85.53***	- 55.75***	+ 148.27***
Muscle	Mean	0.297	0.220	0.188	0.165	0.161	0.152	0.232	0.336
	SD	± 0.011	± 0.013	± 0.009	± 0.005	± 0.002	± 0.008	± 0.016	± 0.008
	% Change		- 25.86***	- 36.70***	- 44.44***	- 45.67***	- 48.54***	- 21.66***	- 13.35***
CNS	Mean	0.112	0.100	0.087	0.038	0.032	0.036	0.101	0.221
	SD	± 0.006	± 0.011	± 0.012	± 0.004	± 0.005	± 0.004	± 0.012	± 0.015
	% Change		- 10.56***	- 21.84**	- 60.01***	- 70.68***	- 67.55***	- 9.82NS	+ 97.61***

*** = P < 0.001; ** = P < 0.01; NS : Not significant.

LEGEND FOR FIGS. 25 TO 28

Histograms showing day-to-day per cent changes in the levels o **trehalase** activity in the haemolymph (Fig.25), silkgland (Fig.26), muscle (Fig.27) and CNS (Fig.28) from the 2nd day to the 7th day of the 5th instar and also on the 1st day of the pupa in *Bombyx mori*. The level on the 1st day was taken as the control (C) for calculating the change on the remaining days.

Fig.25

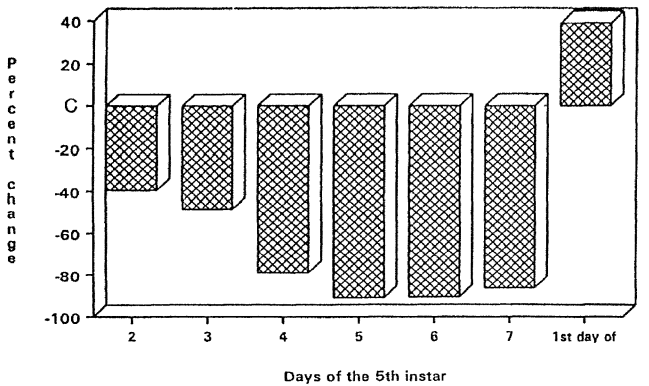


Fig.26

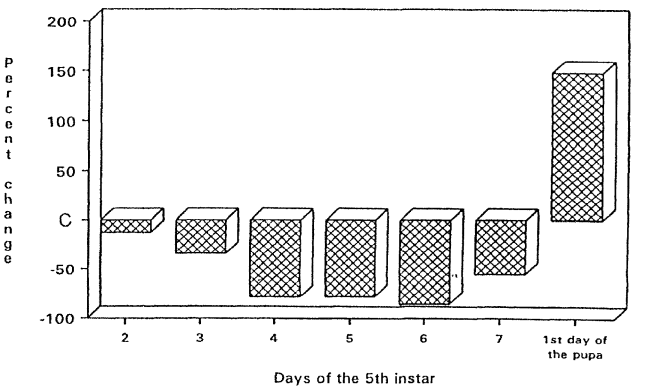


Fig.27

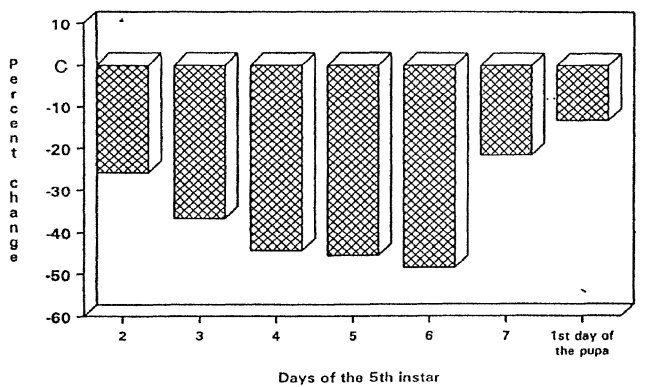
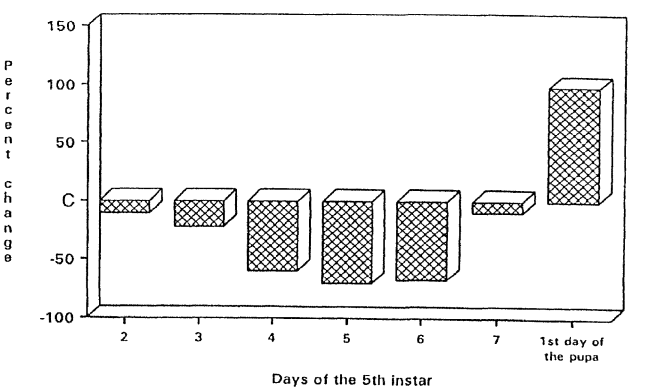
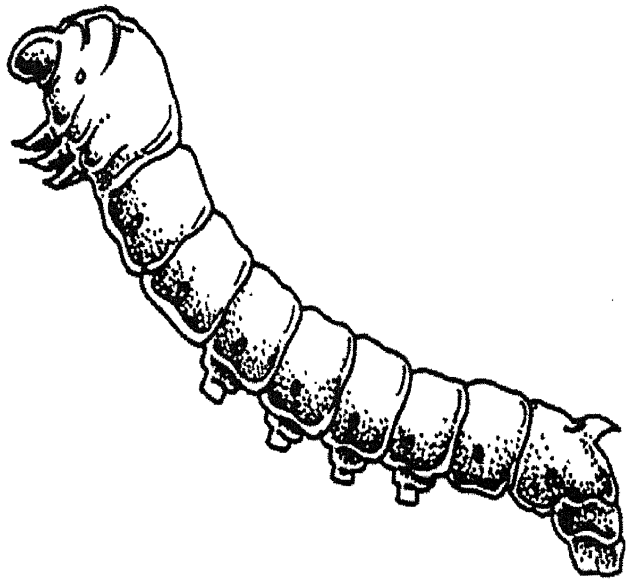


Fig.28



Chapter - II



Biogenic Amines

The monoamines belong to the wider group of neurotransmitters possessing a single amine (NH_2) group. Catecholamines containing a nucleus of catechol (a benzene ring possessing 2 adjacent hydroxyl groups) and a side chain of ethylamine or one of its derivatives are members of this group of neurotransmitters. The important catecholamines are dopamine (DA), nor-epinephrine (NEP) and epinephrine (EP).

In insects, corpora cardiaca are the principal neurohemal organs. These glands both store and secrete neurosecretory products synthesized in the brain and also contain intrinsic cells which produce their own neurosecretory material [Singh *et al.* 1982; Gossey and Candy 1982]. In corpus cardiacum of locusts, biogenic amines have been detected by a radio chemical enzymatic assay and high performance liquid chromatography (HPLC) [Orchard *et al.* 1986].

The catecholamines DA, NEP and EP are neurotransmitters in a number of brain areas subserving functions relating to emotion, attention and visceral regulation and also participate in the control of feeding and body weight regulation [Leibowitz 1992].

The neurotransmitter is present in nerve terminals and it is released upon nerve stimulation. Catecholamine release occurs through a Ca^{2+} - dependent, exocytotic mechanism. The content may be demonstrated by chemical measurement, histofluorescence or immunological markers.

The catecholamines EP, NEP and DA are formed in the brain, chromaffin cells, sympathetic nerves and sympathetic ganglia from their amino acid precursor tyrosine by a sequence of enzymatic steps. These enzymes are tyrosine hydroxylase, dopa-decarboxylase and dopamine-ß-hydroxylase. Certain drugs like vinblastin, cytochalsin beta, and the absence of calcium prevent the release of the transmitters.

Nor-epinephrine is a neurotransmitter associated with emotional states and motor hyperactivity besides other functions. Eluer [1956] predicted that NEP was highly concentrated in the nerve terminal region from which it was released to act as a neurotransmitter. Ephinephrine is also a neurotransmitter but its concentration is more in adrenal medulla than in brain. Dopamine is a transmitter in the brain associated with movement, behaviour and motor function. In addition to its role as a neurotransmitter, DA also serves as the precursor for NEP and EP. The pathways of these catecholamines are separate and distinct. The discovery of serotonin in the nervous system naturally raised the possibility that the compound might serve as a neurotransmitter. Welsh [1957] proposed a neurohumoral role for this substance in invertebrates.

The presence of catecholamines and 5-hydroxytryptamine (5-HT) has been proved by chemical and histochemical methods in several insects. [Gersch *et al.* 1961; Colhoun 1963; Frontali and Norberg 1966; Mancini and Frontali 1970; Bjorklund *et al.* 1970]. The catecholamine content of isolated brain of *Periplaneta americana* was measured by Frontali and Haggendal [1969], whereas that of the thorax of *Anabolia nervosa* was measured by Bjorklund *et al.* [1970].

Attention has been focussed recently on the functional role of central aminergic systems in insects. The catecholamines, DA and NEP, 5-HT and phenolamine octopamine have been detected in the brain of many insects [Mercer and Gupta 1987]. The distribution of these amines throughout the brain is extensive. The presence and role of the biogenic amines in insect development, changes in the concentration of octopamine, DA, EP, NEP and serotonin in the brain has also been shown by Awad *et al.* [1997].

Recently, a wide range of biogenic amines and related metabolites were quantified in CNS and haemolymph of the silkworm *Bombyx mori*, from the 4th instar to the post-spinning stage [Takeda *et al.* 1991]. Madhukar and Rao [1989] studied the effect of certain pharmacological agents on *Bombyx mori* heart. The uptake and release characteristics of DA and serotonin in the salivary glands of *Locusta migratoria* were also examined by Ali and Orchard (1996). The levels of aromatic amino acids and amines in the haemolymph of the larvae of *Mythimna separata* were analysed after parasitization by *Apanteles kariyai* [Shimizu and Takeda 1994]. Changes in the concentration of three catecholamines (EP, NEP and DA) in the brain-suboesophageal ganglion complex were examined during the last larval and early pupal stages of *Mamestra brassicae*, in relation to the induction of pupal diapause [Takeda *et al.* 1994].

Catecholamines are important metabolites for the sclerotization and melanization of insect cuticle [Brunet 1980]. Dopamine has been identified in insects [Ostlund 1954]. It is involved in the hardening of the cuticle [Hackman 1964], but it is also a precursor of NEP and EP [Sekeris and Karlson 1966]. All these catecholamines have some effects on the insect CNS [Hodgson and Wright 1963].

Serotonin (5-hydroxytryptamine, 5-HT) has been proposed to act as a neurotransmitter in the central nervous system [Brodie and Shore 1957]. In invertebrates some roles of serotonin are well known. For example, in the case of fresh water mussel it has a role in nervous control of periodic activity [Salanki 1963], also in the contraction of molluscan smooth muscle [Twarog 1954; 1960] and in ciliary activity of *Mytilus* gill [Aiello 1957]. Serotonin has also been proposed to act as a neurohumoral agent in the lower animals [Welsh 1957]. It has been reported that nerve cells showing immunoreactivity to serotonin are widely distributed in many invertebrate phyla [Fujii and Takeda 1987]. It has been established that during undisturbed conditions, the crustacean nervous system has been shown to contain only small quantities of biogenic amines [Aramant and Elofsson 1976; Elofsson *et al.* 1982].

The available literature shows that very little attention has been focussed on the silkworm, *Bombyx mori* with reference to biogenic amines. A few works are available on the neurotransmitter acetylcholine and its hydrolyzing enzyme acetylcholinesterase, the changes in which during metamosphosis of the silkworm were correlated with the activity for preparation and execution of the spinning phase [Siva Prasad *et al.* 1992; Rajeswari 1994].

The present investigation was undertaken to examine the presence and possible changes in the biogenic amines during the 5th instar of the silkworm *Bombyx mori* and to strike a correlation betweeen these changes and the changing phases of activity during the 5th instar.

METHODS

Extraction and Estimation of Biogenic Amines

Epinephrine (EP), nor-epinephrine (NEP) and dopamine (DA) contents were estimated by the method of Kari *et al.* [1978].

Tissue samples were homogenized in ice-cold acid butanol to give a final concentration of 50 mg/ml. The homogenates were centrifuged at 1000 x g for 15 min at 4°C. The residues were discarded and to the supernatant 2.5 ml of distilled water and 2.5 ml of n-heptane were added. The contents were thoroughly mixed and centrifuged at 1000 x g for 5 min. The aqueous phase was separated and to this 200 mg acid-washed alumina was added, followed by 1.5 ml of 2M sodium acetate. The pH was adjusted to 8.0 using 1 N sodium hydroxide. The samples were again centrifuged at 1000 x g for 5 min. The supernatant (1.5 ml) was collected and used for the estimation of serotonin (5-HT). Cateholamines were extracted from the alumina.

The alumina was washed twice with 2 ml of distilled water by vortexing the tube and centrifuged at 1000 x g for 5 min. The supernatant was discarded and the walls of the tube were blotted with strips of filter paper.

The monoamines were eluted by treating the alumina with 2 ml of 2N acetic aid. The tubes were centrifuged at 1000 x g for 5 min. The supernatant was transferred to another tube. To this 0.1 ml of EDTA was added and the

pH was adjusted to 6.3. Iodine (0.1 ml- 0.1 N iodine in ethanol) was added to the above tube and mixed thoroughly. The samples were allowed to stand at room temperature for 2 min. Then 0.2 ml of alkaline sulphate solution was added. The contents were shaken well and allowed to stand at room temperature for 2 min.

At the end of 2 minutes, the pH of the solution was adjusted to 5.4 with 5N acetic acid. Along with the test samples, tissue samples with known amount of different amine standards were maintained which served as internal standards.

The fluorescence of epinephrine was read in a Perkin-Elmer 3000 fluorescence spectrometer with excitation and emission wavelengths of 410/500 nm respectively, with a slit width of 10 nm.

Norepinephrine was estimated after heating the same solution for 2 minutes in a boiling water bath. The tubes were cooled and the fluorescence of NEP was read in a Perkin-Elmer 3000 fluorescence spectrometer with excitation and emission wavelengths of 385/485 nm respectively, with a slit width of 10 nm.

After the estimation of NEP, the solution was used for the assay of DA. The solution was heated for 5 minutes in a boiling water bath. The tubes were cooled and the fluorescence of DA was read in a Perkin-Elmer 3000 fluorescence spectrometer with excitation and emission wavelengths of 320/370 nm respectively, with a slit width of 10 nm.

Serotonin (5 HT)

After trapping catecholamines from the tissue sample into alumina, the supernatant (1.5 ml) was separated for the estimation of 5-HT. To this supernatant 0.1ml of cysteine, 1.5ml of HCl and 0.1ml of 0-phthalaldehyde (OPA) solution was added. The tubes were kept at room temperature for 20 minutes. Then sodium metaperiodate (0.1ml) was added and the tubes were heated at 80°C in a boiling water bath for 20 minutes. The samples were cooled and the flourescense of serotonin was read in a spectophotometer (Model : RF-500) at excitation and emission wavelengths of 360 and 470 nm respectively with a slit width of 20/10 nm. The amount of serotonin was calculated by the method of Ansell and Besson [1968] and expressed in µg/g wet wt of tissue.

RESULTS

Day-to-day changes in the levels of biogenic amines, viz., epinephrine (EP), norepinephrine (NEP), dopamine (DA) and also 5-Hydroxytryptamine (5-HT) were examined in two tissues, namely central nervous system (CNS) and haemolymph on each of the seven days of the 5th instar and on the 1st day of the pupa. Of these, only NEP, DA and 5-HT were detected, but epinephrine was not detected. The results are given in Tables 8 to 10.

The first day of the 5th instar was taken as the reference point in the experiments and the percent changes in monoamine and 5-HT levels day-to-day as well as statistical significance were calculated by taking this reference point as the control.

Nor-epinephrine

The results on NEP are presented in Table-8; Figs.29 and 30. CNS recorded a higher level of NEP compared to the haemolymph.

In CNS, NEP was not detected from the 1st day to the 4th day of the 5th instar. It was traced on the 5th day and then it showed a gradual increase up to the 1st day of the pupa. In the haemolymph, it was found on all days of the 5th instar and also on the 1st day of the pupa. The NEP content gradually decreased during the 5th instar and then increased on the 1st day of the pupa. Higher overall increases were noticed in the CNS on the 1st day of the pupa [+1155.63%], with the content on the 5th day as the reference point. In the haemolymph greater percent decrease was noticed on the 7th day of the 5th instar [-68.30%]. The changes in the levels of NEP content were not statistically significant on the 2nd day of the 5th instar for haemolymph. The changes on all other days for both the tissues were statistically significant.

Dopamine

Changes in dopamine levels are presented in Table-9; Figs.31 and 32. The dopamine content showed a gradual increase in CNS from the 1st day to the 7th day of the 5th instar and also on the 1st day of the pupa. In the haemolymph, the dopamine content showed elevation from the 1st day and reached maximum on the 7th day of the 5th instar and then drastically decreased on the 1st day of the pupa. Higher percent increases were noticed

on the 1st day of the pupa in CNS [+145.13%]. Higher percent increases were recorded on the 7th day of the 5th instar in haemolymph [+402.38%]. Higher dopamine content was observed in CNS when compared to the haemolymph. The changes in the tissues were not statistically significant on the 2nd day of the 5th instar in CNS, and on the 1st day of pupa in haemolymph. The changes on all the remaining days were statistically significant.

5-HT

5-HT (serotonin) levels examined in CNS and haemolymph are presented in Table-10 and Figs.33 and 34. In the CNS, the 5-HT levels gradually increased during the 5th instar and decreased on the 1st day of the pupa. In the haemolymph 5-HT was not detected in the early 5th instar and it appeared only on the 4th day of the 5th instar. Thereafter it increased slightly up to the 1st day of the pupa. However, the level of 5-HT in haemolymph was very low. Higher percent increases were recorded in haemolymph [+424..48%] followed by CNS [+396.88%]. The changes on all the days for these two tissues were statistically significant.

DISCUSSION

Biogenic amines are neuroactive substances that are widespread in the animal kingdom. The concentration of biogenic amines has been known to vary around the day, seasonally and with developmental stages and stresses [Brown and Nestler 1985; Klemm 1985; Shimizu and Takeda 1991]. It has been suggested that in insects these biogenic amines function as

neurohormones, neurotransmitters and neuromodulators [Evans 1980; Brown and Nestler 1985; Klemm 1985; Orchard *et al.* 1993]. By using HPLC biogenic amine systems have been examined phylogenetically for the establishment of the evolution of chemical communication in the CNS of invertebrates [Takeda 1989].

Major biogenic amines, such as dopamine (DA), norepinephrine (NEP), epinephrine (EP) and 5-hydroxytryptamine (5-HT), play an important role in the regulation of the nervous system in both vertebrates and invertebrates. Among them, catecholamines which are distributed widely in the central nervous system, and help release hormones from the endocrine systems, modulate the energy metabolism which is primarily regulated by the endocrine system, and mediate cellular differentiation via adenylate cyclase [Brown and Nestler 1985].

In the present study the levels of NEP, DA and 5-HT (sentonin) were detected in the central nervous system and haemolymph of the 5th instar larva and also on the 1st day of the pupa of *Bombyx mori*.

EP was not detected in the present study. It has been detected in snails, slugs and crayfishes by HPLC [Takeda 1989, unpublished data]. In *Bombyx mori*, NEP and EP were reported to be present [Drease *et al.* 1960], although the peak purity was unknown. In the present study the biogenic amines could not be estimated using HPLC due to non-availability of this facility. Neverthless, from the spectrofluorometric determination made in the

present study, it does appear that EP was absent in the nervous system and haemolymph during the 5th instar of *Bombyx mori*. This appears to be the consequence of non-conversion of NEP (the precursor) to EP. Presumably EP has no perceptible physiologial role during the 5th instar.

In CNS, NEP was not detected in the early stages of the 5th instar, and it only appeared on the 5th day and thereafter increased up to the 1st day of the pupa. In haemolymph NEP was higher in the beginning of the 5th instar and thereafter showed decreasing trend and again increased towards the end of the 5th instar and also on the 1st day of the pupa. Since the precursor for NEP, i.e. dopamine, is present continuously in the nervous system as well as haemolymph throughout the 5th instar, it is interesting that NEP makes its appearance only from the 5th day in the nervous system. This probably has a special physiological significance in terms of neural activity for the ensuring spinning activity, since NEP presumably can be a neuromodulator in the nervous system of the silkworm.

DA in the CNS increased during the 5th instar and also on the 1st day of the pupa. In the brain of *Bombyx mori*, from larval to adult stages, maximum level [27.6 ng/brain] of DA was found at the post-spinning stage [Takeda *et al.* 1991]. This peak in levels of DA was suggested to be involved in spinning behaviour at the pharate pupal stage. Similar increasing levels were noticed in *Manduca sexta* [Granger *et al.* 1986]. However, in haemolymph DA increased from the 1st day to the 7th day of the 5th instar and decreased on the 1st day of the pupa. This is in consonance with the reports of Brunet [1980], where the levels of DA in the haemolymph prior to

pupation were elevated, a change undoubtedly associated with cuticular sclerotization. In *Mythimna separata* also DA in the haemolymph of unparasitized last instar larvae increased before pupation [Shimizu and Takeda 1994]. DA is involved in the hardening of the cuticle [Hackman 1964], and it is also a precursor of NEP and EP [Sekeris and Karlson 1966]. The continuous presence of DA both in the nervous system and haemolymph compared to NEP and EP probably makes it an important candidate both as the precursor for NEP and EP as well as for a putative role as a neurotransmitter in the silkworm.

The DA level in CNS during the 5th instar was considerably higher than that of the other two biogenic amines (NEP and 5-HT). The highest content of biogenic amines on the 7th day in the CNS presumably indicates increased neuronal activity to aid in the spinning activity. It is to be ascertained as to how far DA is actively involved in neuronal transmission and consequent control of behaviour in *Bombyx mori*.

The concentration of 5-HT showed an increasing trend during the 5th instar and decreased on the 1st day of the pupa in CNS. In the blowfly *Calliphora erythrocephala* during post-embryonic development, the amount of 5-HT detected in the CNS of the newly hatched larvae doubles in a few hours, then remains stable during the first 20-25% of larval life. Then the 5-HT levels begin to increase steadily and reach a maximum at the end of the post-feeding stage. This period of increase covers most of the instar, during which the feeding larvae grow to final size, stop feeding and enter the post-feeding stage. At the end of this stage the mature larvae become less and less active and prepare for pupation [Cantera and Carlson 1988].

The onset of metamorphosis is characterised by a high level of serotonin in the CNS, followed by a decrease. The decrease on the 1st day of the pupa corresponds with the histolysis and general organization of the nervous system. Serotonin is known to control the activity of the salivary glands in *Calliphora* and other physiological and behavioural aspects related to feeding [Nessel 1987].

In the haemolymph 5-HT was not detected on the 1st three days of the 5th instar and it appears only at the middle of the instar and thereafter increases up to the 1st day of the pupa. However, the values are very low, indicating relatively low release or diffusion from the tissues into the haemolymph. In terms of neural activity, the release of 5-HT into the haemolymph at later stages, i.e. a few days before spinning may have some significance in feedback modulation of nervous activity to aid in the spinning behaviour. This assumption requires consolidation.

Although haemolymph is a non-secretory tissue, it contains many biogenic amines. Several biogenic amines such as L-Dopa, DA, NEP, tyramine and 5-HT were detected and their levels estimated in the haemolymph and CNS both in *Bombyx mori* and *Mamestra brassicae* [Shimizu and Takeda 1991; Takeda *et al.* 1991]. Haemolymph has been known to deliver some of these amines and their precursors to certain organs for storage [Evans 1980; Orchard 1982]. Biogenic amines have been shown to be secreted from *Corpora cardiaca*, which contains catecholamines and 5-HT of cerebral origin, into the haemolymph in some locusts and cockroaches [Klemm and Falck 1978; Gresch *et al.* 1974].

One of the characteristics of the insect haemolymph is that it contains many amino acids. Many isolated amino acids including tyrosin have been known to be secreted from the fatbody into the haemolymph. Many enzymes, including phenol oxidase, which act on tyrosine to induce DOPA or DOPA decarboxylase which induce DA, have been reported in the haemolymph of insects [Takeda *et al.* 1991]. Pre-phenol oxidase was also secreted from the fatbody to the haemolymph [Ashida and Ohinishi 1967]. These enzymes become particularly active at metamorphosis after being liberated by the disintegrating tissues [Kuwana 1940].

However, haemolymph is only a transporting medium and not a metabolic site for the biochemical constituents. The presence of catecholamines and 5-HT in the haemolymph could only be due to the leakage or release from other tissues. This explains the lower level of catecholamines and 5-HT in the haemolymph in the present investigation.

Little is known about the functional significance of catecholamines in the ventral nerve cord or brain of insects. Pharmacological actions have implicated DA in behavioural responses of honeybees. DA reduces conditional responses to olfactory stimuli and inhibits information retrieval involved in learning [Mercer and Erber 1983]. In *Drosophila*, a mutant which does not synthesize DA or 5-HT fails to learn in either positively or negatively reinforced olfactory discrimination tests [Livingstone and Tempel 1983].

Biogenic amines have been suggested to serve as releasing factors for peptide hormones in several invertebrate neurosecretory systems [Evans 1980]. Recently, the pituitory thyrotrophic hormone (PTTH) secretion from 2 brain complex as was shown to be stimulated by an indole amine, serotonin (5-HT), via acetylcholine in *Bombyx mori* larvae [Yamada *et al.* 1993]. The

presence of DA cells in the brain and suboesophageal ganglion of the larva and pupa in *Bombyx mori* has been demonstrated immunochemically [Takeda *et al.* 1985]. These cells are located very closely to the PTTH secretory cells at the lateral part of brain [Mizoguchi *et al.* 1990b] and to bombyxin cells in the pars interscerebralis [Mizoguchi *et al.* 1990a].

In *Bombyx mori*, from the 4th instar to the post-spinning stage, the levels of biogenic amines and precursors increased in general both in the brain and suboesophageal ganglion with development [Takeda *et al.* 1991]. By changing the levels of biogenic amines or their precursors the insect might be physiologically able to resist some stresses. DA, NEP and 5-HT were detected in *Schistocerca gregaria* [Robertson 1976] and DA and 5-HT were detected in *Periplaneta americana* [Owen *et al.* 1987]. In most animals except protozoa DA and 5-HT were constantly present in the nervous system. Krueger *et al.* [1990] showed the presence of DA and related substances in the brain and CNS of the larval and adult tobacco hornworm, *Manduca sexta*, and examined their synthetic pathways and physiological roles.

Feeding behaviour logically should be under the control of the nervous system. The functioning of the nervous system depends upon its anatomical organization on one hand and the associated physiological efficacy on the other. The physiological efficacy of synaptic transmission and motor control of tissues would also comprise the neurotransmitters and their changes in the nervous system. The changes in biogenic amines as noticed in the present study in the nervous system and haemolymph during the 5th instar throw some light on their participation in neuronal activity and its involvement in the spinning behaviour in the silkworm *Bombyx mori*.

Table-8 : Changes in the levels of **nor-epinephrine** [NEP] during the 5th instar development of *Bombyx mori*. Each value, expressed as µg/g wet wt of the tissue or 1 ml of haemolymph, is the mean ± standard deviation (SD) of six separate observations. For each observation tissue from about 50 larvae was pooled. Percent changes for all days were calculated taking the 1st day of the 5th instar as the reference point. For nervous system the 5th day was taken as the reference point, since before that day the transmitter could not be detected.

Name of the tissue		Days of the 5th instar							1st day of the pupa
		1	2	3	4	5	6	7	
Nervous system	Mean	-	-	-	-	0.11	0.51	1.04	1.38
	SD					±0.05	±0.19	±0.13	±0.12
	% change						+361.63**	+845.09***	+1155.63***
Haemolymph	Mean	0.13	0.10	0.08	0.07	0.06	0.04	0.04	0.09
	SD	±0.03	±0.01	±0.02	±0.01	±0.01	±0.01	±0.01	±0.01
	% change		-22.47^{NS}	-37.20**	-44.34***	-51.04***	-66.66***	-68.30***	-28.57**

*** P < 0.001; ** P < 0.01; NS : Not Significant

LEGEND FOR FIGS. 29 AND 30

Histograms showing day-to-day per cent changes in **norepinephrine** content in the haemolymph (Fig.29) and CNS (Fig.30) from the 2nd day to the 7th day of the 5th instar and 1st day of the pupa in *Bombyx mori*. The level on the 1st day was taken as the control (C) for calculating the change on the remaining days. For nervous system, the 5th day was taken as the reference point, since before that day the transmitter could not be detected.

Fig.29

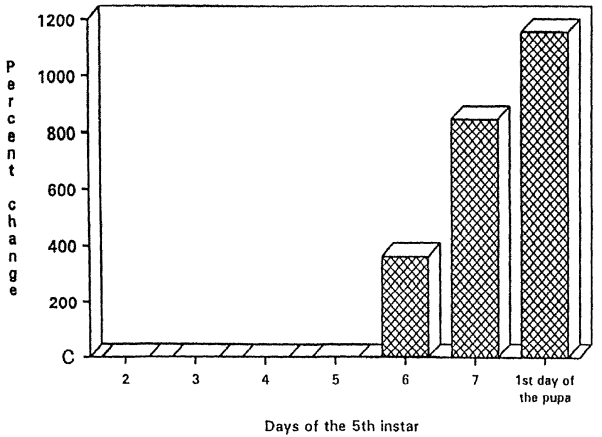

Percent change
1200
1000
800
600
400
200
C
2
3
4
5
6
7
1st day of the pupa
Days of the 5th instar

Fig.30

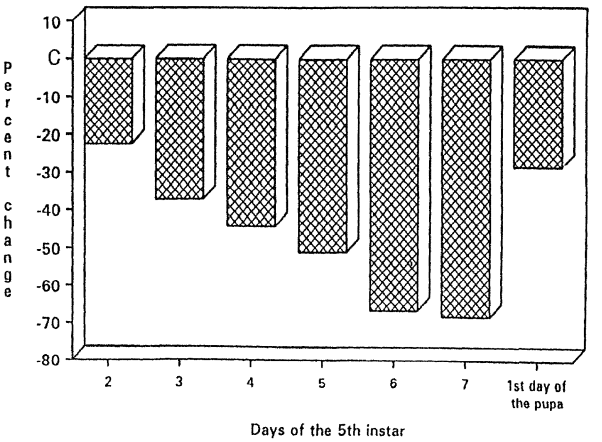

Percent change
10
C
-10
-20
-30
-40
-50
-60
-70
-80
2
3
4
5
6
7
1st day of the pupa
Days of the 5th instar

Table-9 : Changes in the levels of **dopamine** (DA) during the 5th instar development of *Bombyx mori*. Each value, expressed as µg/g wet wt of the tissue or 1 ml of haemolymph, is the mean ± standard deviation (SD) of six separate observations. For each observation tissue from about 50 larvae was pooled. Percent changes for all days were calculated taking the 1st day of the 5th instar as the reference point.

Name of the tissue		Days of the 5th instar							1st day of the pupa
		1	2	3	4	5	6	7	
Nervous system	Mean	1.34	1.60	1.92	2.56	2.56	2.68	2.71	3.29
	SD	±0.27	±1.11	±0.16	±0.42	±0.48	±0.53	±0.53	±0.43
	% change		$+19.06^{NS}$	$+43.16^{***}$	$+90.64^{***}$	$+99.38^{**}$	$+99.40^{**}$	$+101.36^{**}$	$+145.13^{***}$
Haemolymph	Mean	0.15	0.22	0.38	0.39	0.51	0.64	0.76	0.27
	SD	±0.02	±0.02	±0.08	±0.02	±0.06	±0.06	±0.11	±0.06
	% change		-48.67^{**}	-151.98^{***}	$+162.56^{***}$	$+237.04^{***}$	$+326.58^{***}$	$+402.38^{***}$	$+79.49^{NS}$

*** $P < 0.001$; ** $P < 0.01$; NS : Not Significant

LEGEND FOR FIGS. 31 AND 32

Histograms showing day-to-day per cent changes in **dopamine** in the haemolymph (Fig.31) and CNS (Fig.32) from the 2nd day to the 7th day of the 5th instar and also on the 1st day of the pupa in *Bombyx mori*. The level on the 1st day was taken as the control (C) for calculating the change on the remaining days.

Fig.31

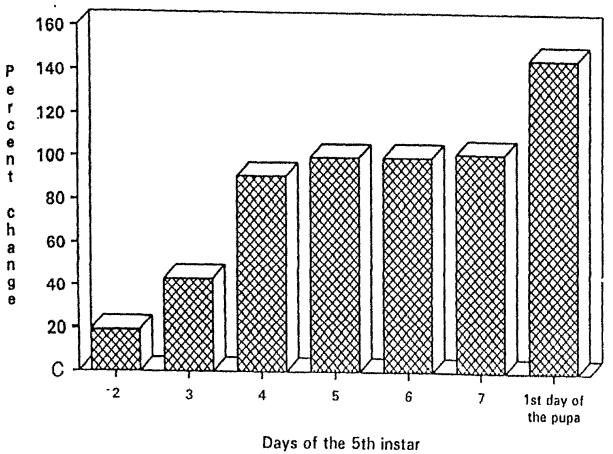

160
140
120
100
80
60
40
20
C
Percent change
2
3
4
5
6
7
1st day of the pupa
Days of the 5th instar

Fig.32

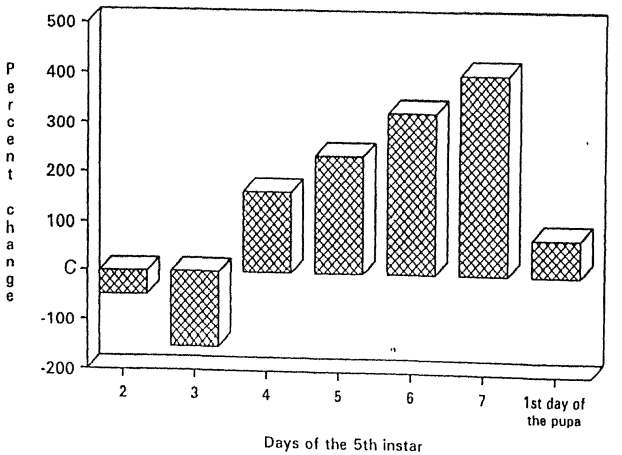

500
400
300
200
100
C
-100
-200
Percent change
2
3
4
5
6
7
1st day of the pupa
Days of the 5th instar

Table-10 : Changes in the levels of **serotonin** [5-HT] during the 5th instar development of *Bombyx mori*. Each value, expressed as µg/g wet wt of the tissue or 1 ml of haemolymph, is the mean ± standard deviation (SD) of six separate observations. For each observation tissue from about 50 larvae was pooled. Percent changes for all days were calculated taking the 1st day of the 5th instar as the reference point. For haemolymph the 4th day was taken as the reference point, since before that day the transmitter could not be detected.

Name of the tissue		Days of the 5th instar							1st day of the pupa
		1	2	3	4	5	6	7	
Nervous system	Mean SD % change	0.42 ±0.11	0.63 ±0.13 +50.04*	0.88 ±0.12 +107.98**	0.99 ±0.13 +134.45***	1.40 ±0.21 +231.47***	1.67 ±0.12 +296.83***	2.10 ±0.22 +396.88***	1.15 ±0.09 +170.79***
Haemolymph	Mean SD % change	- -	- -	- -	0.01 ±0.002	0.02 ±0.00 +177.558**	0.03 ±0.007 +273.46**	0.04 ±0.01 +346.93***	0.05 ±0.01 +424.48**

*** $P < 0.001$; ** $P < 0.01$; * $P < 0.05$.

LEGEND FOR FIGS. 33 AND 34

Histograms showing day-to-day per cent changes in **serotonin (5-HT)** in the haemolymph (Fig.33) and CNS (Fig.34) from the 2nd day to the 7th day of the 5th instar and also on the 1st day of the pupa in *Bombyx mori*. The level on the 1st day was taken as the control (C) for calculating the change on the remaining days. For haemolymph, the 4th day was taken as the reference point, since before that day the transmitter could not be detected.

Fig.33

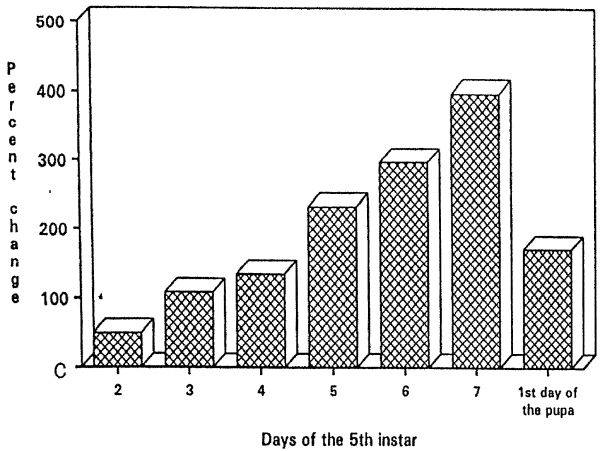
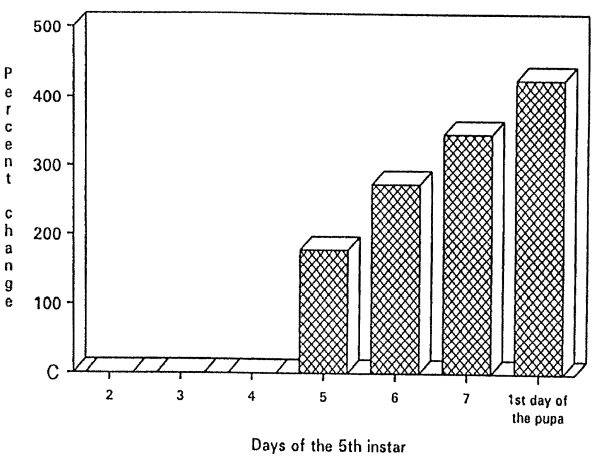
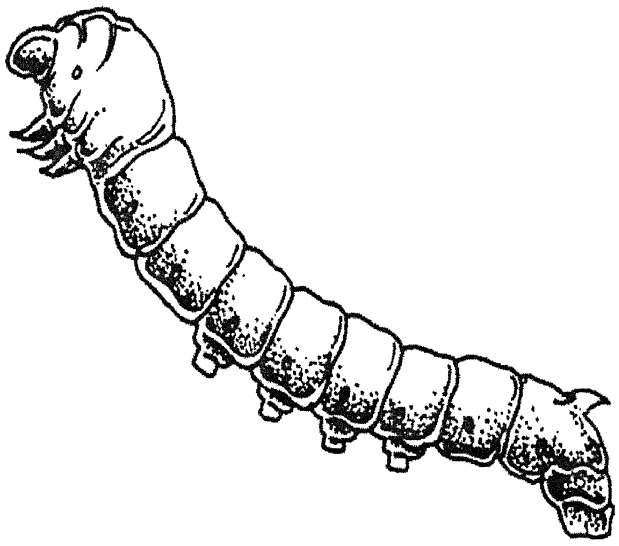

Percent change
500
400
300
200
100
C
2
3
4
5
6
7
1st day of the pupa
Days of the 5th instar

Fig.34


Percent change
500
400
300
200
100
C
2
3
4
5
6
7
1st day of the pupa
Days of the 5th instar

Chapter - III



Spontaneous Electrical Activity

INTRODUCTION

Environmental changes (stimuli) may alter the distribution of ions across the cell membrane and create measurable electrical currents. These responses, manifest as deviations in the membrane potential from its value in the unstimulated cell, are termed 'electrogenesis' [Grundfest 1961].

Electrogenesis forms the basis for excitation leading to muscle contraction, nerve conduction, gland secretion and other physiological processes. Experimentally, the electrical properties of cells can be most readily explored in the neurons of invertebrate animals (squids, crabs), and many of the pioneer investigations were carried out with these preparations. However, it is emphasized that electrical signalling is characteristic of many other kinds of cells (muscle fibres, plant cells, gland cells) and a fundamental property of living tissues.

Two types of signals occur in electrogenesis : *Localized* or *electrotonic potentials*, and *action potentials* or *spikes*. Hodgkin (1951) did the classical experiments using large nerve fibres isolated from the limb nerves of the shore crab *Carcinus maenas*.

Electrical activity of the nervous system can be broadly visualised as falling into two categories, viz., (a) the endogenous ongoing activity or the spontaneous activity which does not require specific stimuli other than the natural changes in the external environment and the accompanying changes in the internal milieu, and (b) action potentials that are evoked by specific stimuli. Both forms of activity can be modified experimentally, using various agents, *in vitro* as well as *in vivo*.

Spontaneous electrical activity in the central nervous system was first demonstrated by Adrian [1931] in caterpillar and water beetle, and is now known to occur in all groups of animals. Amongst the arthropods, spontaneous activity was well studied in crustaceans and insects [Bullock and Horidge 1965]. Van Der Kloot (1955) reported that the CNS shows a significant change in its electrical activity during metamorphosis of certain lepidopteran insects. According to him, electrical activity which is characteristic of the larval brain disappears before the pupal molt, and the latter becomes electrically silent in the pupa, while the ventral ganglia retained it throughout the diapause. He also observed that the brain of the 5th instar larva which was endocrinologically and electrically active became electrically silent in the pupa. Contrary to this view, Schoonhoven (1963) reported that the spontaneous electrical activity in the brain of several lepidopteran and other diapausing insects did not disappear completely. On the other hand Tyshtchenko and Mandelstam (1965) have demonstrated that the electrical activity of the pupal nerve chain was depressed in the oak silkworm, *Antheraea*. Similar inhibition in the bioelectric activity of pupal nerve chain of 10 lepidopteran species was reported [Tyshtchenko 1964].

Spontaneous activity of the CNS is known to be influenced by neurohormones [Ozbas and Hodgson 1958; Weiant 1958; Milburn, Weiant and Roeder 1960]. Strejckova *et al.* [1965] demonstrated the occurrence of two separate neurohormones in the brain and corpora cardiaca of the cockroach, one capable of evoking hyperautorhythmia in the CNS and the other capable of inhibiting the existing electrical activity in the ganglia. Investigation on the electrical activity of neurosecretory cells of *Bombyx mori* brain was reported by Miyazaki and Shun-Ichi [1980].

Effects of a wide variety of neurotransmitters and precursors have been tested in a series of studies [Schantz and Goyffon 1970; Goyffon *et al.* 1980; Goyffon and Niaussat 1975; Goyffon and Vachon 1976]. On the basis of electrophysiological data the transmitter role of monoamines was suggested in the CNS of some insects [Florey 1967]. Recently, Madhusudhana [1995] studied the effects of different transmitters such as epinephrine, norepinephrine, dopamine and 5-HT, and of drugs such as imipramine, picrotoxin and reserpine on the spontaneous activity.

The nervous system coordinates and controls the various organs of the body through neural signals and changes in its electrical activity. Increased activity of the sex organs and oviposition [Yamaoka and Hirao 1973, 1977], the spiracular muscles [Van Der Kloot 1963] etc. are controlled by the electrical activity of the nervous system. Ruegg *et al.* [1982] demonstrated that ovarectomy of the adult female results in a reduction in the electrical activity of corpus cardiacum. Andrez *et al.* [1985] studied the directional vibratory signals in the ventral nerve cord of *Locusta*, and concluded that they come from the thoracic ganglia. Siva Prasad *et al.* [1992] observed changes in the spontaneous electrical activity of the ventral nerve cord during the metamorphosis of *Bombyx mori*. Very recently, Varalakshmi [1998] examined the spontaneous electrical activity of the ventral nerve cord in the 2nd day larva and the spinning larva of the 5th instar silkworm. She also studied the effect of central nervous extracts and haemolymph from the 2nd day larva as well as the spinning larva on the spontaneous electrical activity.

Despite the presence of numerous investigations on electrical activity of the nervous system in insects, so far the studies pertaining to the developmental changes in electrical activity and the causes responsible for it have been limited. The present investigation was undertaken to examine the spontaneous electrical activity of the double ventral nerve cord and correlate it with the ongoing morphological and biochemical changes in the CNS during the 5th instar of *Bombyx mori*. Further, the possible influence of neurotransmitters on the spontaneous activity during the 5th instar was also examined.

METHODS

Larvae of the 5th instar of NB_4D_2 hybrid of *Bombyx mori*, reared as described earlier in the section "Material and Genral Methods", were used in the experiments. Depending on the need, the experiments were conducted from the 1st to the 7th day of the 5th instar up to the point when the larvae were just about to start spinning the cocoons.

Preparation of *Bombyx* Ringer

Bombyx Ringer was prepared according to the formula of Yamaoka *et al.* (1971). The following was the composition of the Ringer.

1.	Sodium chloride (NaCl)	8.620 g/l
2.	Potassium chloride (KCl)	0.100 g/l
3.	Calcium chloride ($CaCl_2$)	0.441 g/l
4.	Sodium biphosphate (Na_2HPO_4)	0.597 g/l
5.	Potassium biphosphate (KH_2PO_4)	0.227 g/l

The Ringer was stored in a refrigerator (5°C) and was brought to laboratory temperature before use.

Preparation of neurotransmitter solutions

10^{-1} M solutions of the neurotransmitters, viz., epinephrine (EP), nor-epinephrine (NEP), dopamine (DA) and 5-hydroxytryptamine (5-HT) were prepared in cold *Bombyx* Ringer and stored in a refrigerator. They were brought to laboratory temperature before use and diluted according to the need with *Bombyx* Ringer.

Measurement of spontaneous electrical activity

Fig.35 shows the organization of the central nervous system of *Bombyx mori*. The ventral nerve cord (VNC) was exposed from the dorsal side and the recordings of spontaneous electrical activity were made between SG - 1TG, 1TG - 2TG, 2TG - 3TG, 3TG - 1AG, 1AG - 2AG, 2AG - 3AG and 3AG - 4AG, 4AG - 5AG, 5AG - 6AG and 6AG - 7/8AG. The recordings were made at the same time every day, although in a metamorphosing animal the interference of diurnal variations is unclear. Paired platinum hook electrodes were used to monitor the spontaneous electrical activity. The potentials were fed through a Nihon Kohden AVB-21 preamplifier and displayed on a Nihon Kohden VC-11 memory oscilloscope. Photographic readings were made using a Nihon Kohden RLG-6201 oscilloscope camera.

Effects of neurotransmitters on spontaneous electrical activity

The 7th day larvae of the 5th instar were chosen for the study of the effects of different neurotrasmitters. The effects were examined at 2AG-3AG, since on the 7th day of the 5th instar this segment of VNC was the longest and so manoeuvre with electrodes was easy. Further, it did not make any difference as to from which abdominal segment the activity was recorded, since all abdominal segments had more or less the same level of activity (Plates-III to VI; Tables-3 to 6). Different concentrations of neurotransmitters ranging from $1x10^{-8}$ M to $1x10^{-2}$ M were prepared in *Bombyx* Ringer. Each transmitter was tested for its effect at these concentrations starting from $1x10^{-8}$M and going up. The control activity was recorded first, and then the VNC was soaked for 3 min in $1x10^{-8}$ M solution of the neurotransmitter under question, and the activity was recorded again. Following this the VNC was thoroughly washed with *Bombyx* Ringer for 5 min to check if there was any reversal of the effect to the control level. In this way the spontaneous electrical activity was recorded after treatment with each concentration of the different neurotransmitters, and after washing with Ringer.

Analysis of spontaneous electrical activity

Analysis of spontaneous electrical activity in the control and experimental conditions was made by counting the number of spikes present in a 50 cm long film slip on which the spontaneous activity was recorded. Spikes from recorded film strips from 6 separate experiments were thus counted and the mean was taken. The data was then converted into spikes/sec. The number of categories of spikes firing in the controls and under each experimental condition were approximated basing on their amplitudes.

Spontaneous electrical activity recorded from different segments of the ventral nerve cord (VNC)

The spontaneous electrical activity was recorded from the connectives of the VNC between consecutive ganglia starting from the suboesophageal ganglion up to the last abdominal ganglion on different days of the 5th instar. The activity in the VNC could be recorded as continuous firing of a number of units differing in their amplitudes and frequency. The spikes had a steep ascending and descending contour and looked like unit potentials. These units could be distinguished into about 3 or 10 categories depending upon their amplitudes. The potentials having the same amplitude were reckoned as belonging to one category.

a. Spontaneous electrical activity from 1st day of the 5th instar

The firing of the VNC varied from segment to segment (Plate-I; Table-11). Higher activity was recorded between the suboesophageal ganglion (SG) and the 1st thoracic ganglion (1TG). The activity recorded between SG & ITG had a frequency of about 212 spikes/sec (Plate-I,A; Table-11). Basing on the amplitudes of the potentials 7 spike categories could be identified. The activity decreased in the next segment, i.e. between 1TG - 2TG. It had a frequency of 70 spikes/sec, and the spike categories were only 3. Thereafter the activity increased slightly between 2TG - 3TG and 3TG - 1AG with a frequency of 88 and 125 spikes/sec, and 3 spike categories each respectively.

The activity in the abdominal cord was higher than in the thoracic part. However, the activity between different segments of the abdominal cord was more or less of the same level, with minor differences. As such, only the recordings made form 1AG - 2AG, 2AG - 3AG and 6AG - 7/8AG are shown in the Plate-I to avoid redundancy. Between 1AG - 2AG, 2AG - 3AG and 6AG - 7/8AG the activity recorded had a frequency of 164, 154 and 155 spikes/sec and 6,6 and 4 spike categories respectively.

b. Spontaneous electrical activity from the 7th day of the 5th instar

The spontaneous activity recorded from different segments of the VNC of the 7th day larva of the 5th instar is presented in Plate-II; Table-12. The activity was in fact examined on all days of the instar, from which it was apparent that there is an increase in activity throughout the VNC from the 1st day to the 7th day. However, the trend of variation in activity between different segments of VNC was the same on all the days. In all cases, the activity was highest at SG - 1TG and then decreased at 1TG - 2TG. The abdominal segments of the cord showed a higher level of activity than the thoracic segments, but within the abdominal segments the activity level was more or less the same (Plate-II; Table-12).

The activity recorded between the SG & 1TG had a frequency of 275 spikes/sec, and 9 spike categories were identified. At 1TG - 2TG, 2TG - 3TG, 3TG - 1AG, 1AG - 2AG, 2AG - 3AG and 6AG - 7/8 AG, the activity was found to have frequencies of 103, 157, 162, 241, 228 and 245 spikes/sec and 4,7,5,6,6 and 8 spike categories respectively (Table-12).

Effect of epinephrine (EP)

Treatment of the VNC with EP in general had an elevatory effect (Plate-III; Table-13). The elevation gradually increased from 1 x 10^{-8} M onwards, with maximum elevation occurring with EP concentration of 1x10^{-5} M. Thereafter, further increase in the concentration of EP resulted in a gradual decrease at 1x10^{-4} M and above. Washing the VNC with *Bombyx* Ringer following treatment with each concentration of EP could effect complete recovery to the control (recordings and data not shown).

The control activity recorded at 2AG - 3AG from the 7th day larvae had a frequency of about 213 spikes/sec (Plate-III, A; Table-13). Basing on the amplitudes of potentials the spike categories were discerned as 5. Treatment of the VNC with 1x10^{-8} M EP resulted in an increase of the control firing to 243 spikes/sec, and 6 spike categories were identified. Treatment with 1x10^{-7} M, 1x10^{-6} M, 1x10^{-5} M and 1x10^{-4} M EP resulted in frequencies of 285, 298, 340 and 216 spikes/sec, with 7,8,8 and 5 spike categories identified respectively. Higher percent increase was noticed on treatment with 1x10^{-5}M (+60%), followed by 1x10^{-6} M (+40.09%), 1x10^{-7} M (+33.89%) and 1x10^{-8} M (+14.18%). The activity after treatment with 1x10^{-4} M was about control level only (+1.31%). The elevation with different concentrations from 1x10^{-8} M to 1x10^{-5} M was statistically significant.

Effect of nor-epinephrine (NEP)

Results on the effect of NEP on spontaneous activity are presented in Plate-IV; Table-14. The control activity recorded had a frequency of 210 spikes/sec and 5 spike categories were identified. In contrast, treatment with NEP caused effects which were different from those with NEP. Treatment with $1x10^{-8}$ M NEP caused maximum increase in activity, with a spike frequency of 443/sec and 11 spike categories. Treatment with $1x10^{-7}$ M, $1x10^{-6}$M, $1x10^{-5}$ M and $1x10^{-4}$ M recorded a frequency of 292, 275, 223 and 198 spikes/sec respectively. The spike categories did not differ very much from the controls, in that they were 6, 6, 4 and 6 respectively. Treatment with $1x10^{-8}$ M NEP recorded a greater change of +110.86% from the control. Following this, treatment with $1x10^{-7}$ M NEP caused a decrease of about +39.24% from the control. This decrease continued further up to $1x10^{-4}$ M NEP, when a level of about +5.62% from the control value was recorded (Plate-IV,E; Table-4). Except for the concentrations of NEP of $1x10^{-5}$ M and $1x10^{-4}$ M, the other changes were statistically significant.

Effects of dopamine (DA)

Results on the treatment with different concentrations of DA are presented in Plate-V; Table-15. The control activity recorded had a frequency of 217 spikes/sec, and 5 spike categories were observed. Highest activity recorded on treatment with $1x10^{-6}$ M DA had a frequency of 314 spikes/sec. The spike categories also were found to be higher (9) on treatment with $1x10^{-6}$ M DA. The activity at $1x10^{-4}$ M DA had a frequency of 204 spikes/sec.

Treatment with $1x10^{-8}$ M, $1x10^{-7}$ M DA and $1x10^{-5}$ M DA resulted in frequencies of 248, 265 and 259 spikes/sec, and the spike categories were 6, 8 and 5 respectively. Higher percent increases were noticed on treatment with $1x10^{-6}$ M (+44.88%) followed by $1x10^{-7}$ M (+22.21%), $1x10^{-5}$ M (+19.45%) and $1x10^{-8}$ M (+14.47%) respectively. A decrease was noticed for treatment with $1x10^{-4}$ M DA (-5.81%). Except with the concentration of $1x10^{-4}$ M DA, the other changes were statistically significant.

Effects of 5-hydroxytryptamine (5-HT)

Effects of different concentrations of 5-HT were presented in Plate-VI; Table-16. Recording with Ringer (control) showed a frequency of 214 spikes/sec, and 5 spike categories were noticed. The trend of activity was similar to that of treatment with NEP. The highest activity recorded at a 5-HT concentration of $1x10^{-8}$ M had a frequency of 324 spikes/sec, and 6 spike categories were observed. Following this, treatment with $1x10^{-7}$ M caused a decrease with a spike frequency of 289 spikes/sec, and 6 spike categories were identified. This decrease continued further up to $1x10^{-4}$ M 5-HT, when a decrease in frequency to about 233 spikes/sec and 4 spike categories was recorded. Greater percent increase was noticed on treatment with $1x10^{-8}$ M (+51.45%), followed by $1x10^{-7}$ M (+34.83%), $1x10^{-6}$ M (+29.13%) and $1x10^{-5}$ M (+24.37%) respectively. Little increase was recorded on treatment with $1x10^{-4}$ M (+8.96%). The elevation with different concentrations from $1x10^{-8}$ M to $1x10^{-8}$ M was statistically significant.

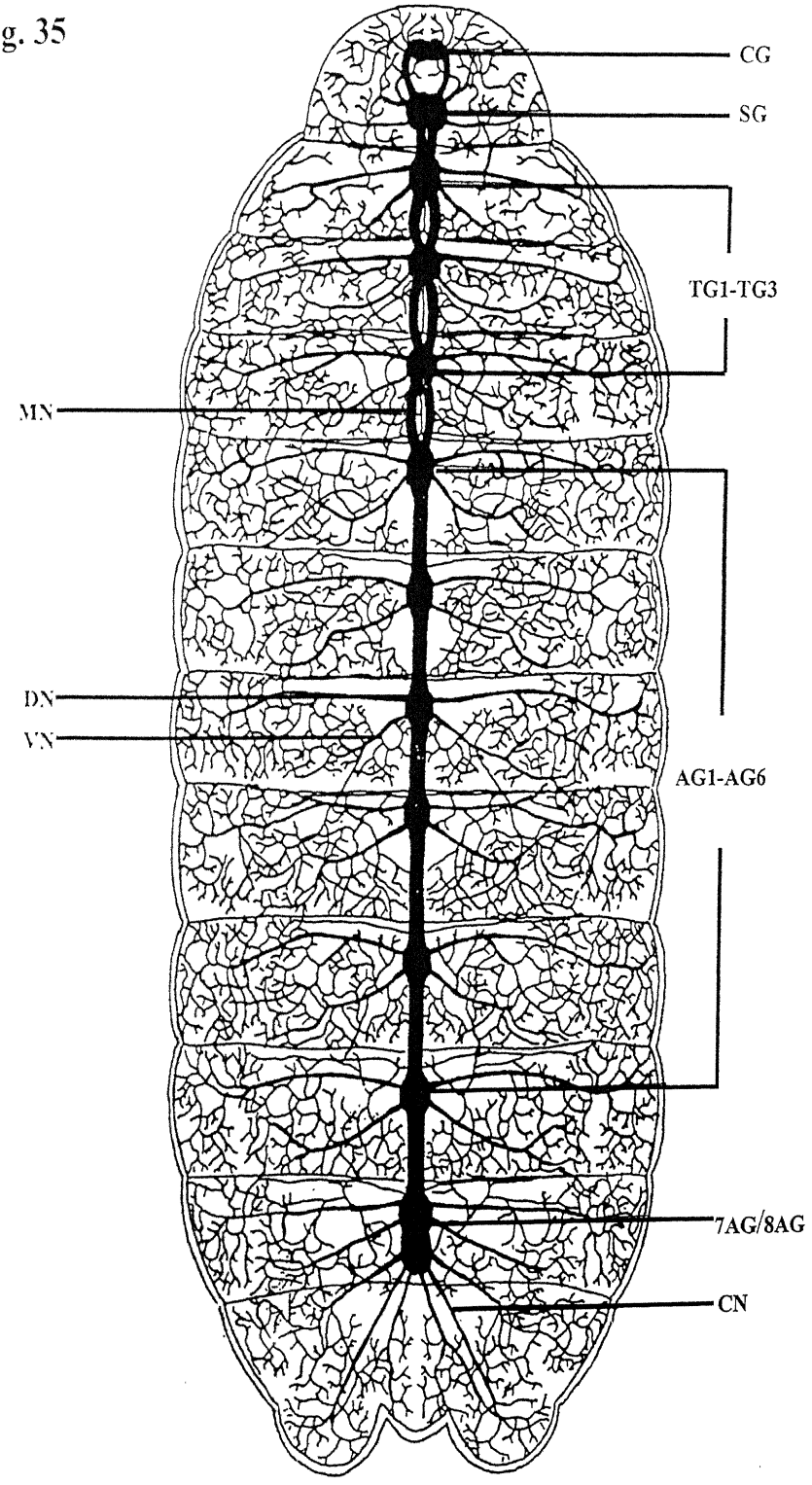
LEGEND FOR FIG. 35

Organisation of central nervous system (CNS) and peripheral nervous system (PNS) of the silkworm *Bombyx mori* by about the 7th day of the 5th instar (From Siva Prasad and Murali Mohan 1998).

CG	-	Cerebral ganglion (Brain).
SG	-	Suboesophageal ganglion.
TG1-TG3	-	1st to 3rd thoracic ganglia.
AG1-AG6	-	1st to 6th abdominal ganglia.
7AG/8AG	-	Fuged 7th and 8th abdominal ganglia.
MN	-	Median nerve
DN	-	Dorsal nerve
VN	-	Ventral nerve
CN	-	Caudal nerve

The spontaneous activity was recorded from all the interganglionic connectives from SG-7AG/8AG. Effects of neurotransmitters was studied at AG2-AG3.

Fig. 35



The above observations lead to the assumption that the overt activity of the silkworm increases as it proceeds from the 1st day to the 7th day of the 5th instar, leading towards the spinning activity. In consonance with this assumption a gradual increase in spontaneous activity in different segments of the VNC was noticed in the present investigation. Siva Prasad *et al.* (1992) reported that the spontaneous electrical activity increases during the 5th instar of the silkworm *Bombyx mori*. Results of the present study are in agreement with this observation.

Siva Prasad (1987) reported that during metamorphosis, the CNS of the silkworm undergoes a series of morphological changes such as an increase in the size of the brain and ventral ganglia, lengthening and shortening and final disappearance of certain ganglionic connectives culminating in the final coalescence of certain ventral ganglia to form anterior and posterior ganglionic complexes. The branching and innervation pattern of the segmental nerves gradually increases during metamorphosis (Fig.35), with the establishment of more and more synaptic contracts with the segmental muscles [Siva Prasad and Murali Mohan 1998]. Thus there is a sort of reorganization in the nervous system in which both the motor and sensory neurons are involved. The increase in the spontaneous activity could be correlated with the increase in the complexity of the nervous system, especially in terms of its peripheral organisation, which enhances peripheral input of information to the CNS.

The activity of the nervous system, whether spontaneous or evoked, would depend on the metabolic changes occurring both in the nervous system and in its environment. The present study has recorded changes in the levels of total carbohydrates, glycogen, glucose and trehalose and in the activities

of glycogen phosphorylase and trehalase in different tissues including the nervous system itself. The changes in biochemical composition of the haemolymph bathing the nervous system could have a bearing on the electrical activity as demonstrated earlier by several investigators [Rao and Gopalakrishna Reddy 1967; Murali Mohan and Sasira Babu 1976; Varalakshmi 1998]. The increase in spontaneous activity from the 1st day to the 7th day of the 5th instar as noticed in the present investigation could be related to these factors in addition to the increase in the complexity of the nervous system, and other intrinsic and extrinsic factors which may play a role.

Gradient in spontaneous activity

In the present study the possible presence of a gradient in spontaneous activity in the nervous system of *B.mori* was also examined. This issue assumes importance in view of the fact that metamorphosis is a dynamic process which entails structural, physiological and functional changes from one stage to the other. In such an event, whether there are specific patterns of activity in the nervous system and, if they are present, whether they are subject to dynamic changes during metamorphosis is interesting to examine. The existence of an antero-posterior gradient in spontaneous activity of the ventral nerve cord [Venkatachari 1971] and of the cardiac ganglion [Devarajulu Naidu 1974] was demonstrated in the scorpion *Heterometrus fulvipes*. An antero-posterior gradient in the spontaneous activity was reported in the crayfish nerve cord [Prosser 1934].

Results of the present study demonstrate a specific pattern of spontaneous activity in the ventral nerve cord, if not a gradient as such. The activity was found to be highest between the suboesophageal ganglion (SG) and 1st thoracic ganglion (TG1). Then the activity decreased in TG1-TG2 and TG2-TG3, thus showing an overall decrease in the thoracic segments. Following this the activity increased in TG3-AG1. From then on the activity remained elevated in the remaining abdominal segments compared to the thoracic segments. There was no gradient in activity in the abdominal segments, and the activity level was more or less similar. Maximum activity was recorded from AG2-AG3. This pattern was essentially the same from the 1st day to the 7th day of the 5th instar, with quantitative changes depending upon the day of the instar, viz., early, middle or late (Plate-I and II; Table-11 and 12).

The above observations indicate a pattern wherein the cerebral part (brain + suboesophageal ganglion) of the nervous system shows higher activity followed by the abdominal cord, with less activity in the thoracic segments. Thus greater peripheral input and central motor output seem to be concentrated in the cephalic region, with the silkworms actively involved throughout in feeding, and from the 7th day onwards during the 5th instar in spinning the cocoon. Higher activity in the abdominal region may probably be attributed to the active abdominal movements and the peripheral sensory input thereof. Higher activity in the abdominal region compared to the thoracic region seems to be a general feature in insects and arachnids.

Tyshtchenko and Mandelstam (1965) reported in oak silkworm that the highest level of spontaneous electrical activity with reference to both frequency and amplitude is present in the abdominal ganglia. Similar observation was also made in the mulberry silkworm [Koshtoyantz *et al.* 1954]. Experiments on spontaneous electrical activity were performed on the abdominal cord in cockroach [Vijayalakshmi *et al.* 1977; Murali Mohan *et al.* 1983; Venkatachari 1971; Jacob Das 1993] owing to the higher activity which could be pharmacologically modified effectively. Further, the activity in the abdominal cord was found to be higher between the 2nd and 3rd abdominal ganglion. Venkatachari (1971) and Jacob Das (1993) working on the scorpion *Heterometrus fulvipes* found higher level of activity between the 2nd and 3rd ganglia and attributed it to greater profusion of fibres between these two ganglia. A similar anatomical situation probably exists in the silkworm, resulting in higher activity in this segment of the cord.

Thus the pattern of activity along the ventral nerve cord seems to have been pre-fixed in the silkworm *Bombyx mori*, with only quantitative changes occurring as the metamorphosis progressed, in line with the profusion of peripheral branches of the nervous system, showing the highest activity by about the time the 5th instar larva starts spinning the cocoon.

Effects of neurotransmitters.

In the present study the effects of selected commonly known neurotrasmitters were examined on the spontaneous activity of the ventral nerve cord (VNC) of *Bombyx mori*. Since quite logically the nervous system

of the silkworm, as that of other animals, could contain multiple transmitter candidates, performing similar or antagonistic functions, it would be of interest to see if these compounds are excitatory, inhibitory or ineffective, to envisage the nature of function each of them might be subserving. Such studies are not available on the silkworm. The presence and excitatory effects of acetylcholine (ACh) in *Bombyx mori* were demonstrated by earlier investigators of our laboratory [Sasikala and Murali Mohan 1998; Varalakshmi 1998]. In the present study the effects of biogenic amines were examined. The insect nervous system is known to contain the catecholamines epinephrine (EP), norepinephrine (NEP) and dopamine (DA), although relatively little is known regarding their physiological role [Evans 1980]. Biogenic amines have been shown to be active in the CNS of different arthropods [Dieter and Horn 1992] and are known to regulate locomotion and feeding behaviour [Rose and Benjamin 1981].

As stated in the foregoing discussion, the spontaneous activity in the abdominal cord was highest between the 2nd and 3rd abdominal ganglia. This segment was also the longest in the VNC of the 5th instar. For these reasons the effects of neurotransmitters were examined in this segment for the facility of manoeuvrability with electrodes and clear visualization of changes in activity with the neurotransmitters.

Norepinephrine

Norepinephrine (NEP) was found to significantly elevate the spontaneous activity of the VNC at a concentration of $1x10^{-8}$ M (Plate-IV;

Table-14). At concentrations higher than this the elevation was less, and at $1x10^{-5}$ M and $1x10^{-4}$ M there was no perceptible effect.

Norepinephrine as a sympathetic neurotransmitter in the vertebrate nervous system is known to be excitatory [Cooper, Bloom and Roth 1982]. Acetylcholine, adrenalin and noradrenalin were shown to have a stimulatory effect on the neurally mediated hindgut contractions in the crab [Florey 1954]. The transmitter was identified and quantitatively measured in the nervous system and heamolymph of the crayfish, and an excitatory role for it was implicated [Elofsson *et al.* 1982]. However, Goyffon (1978) reported inhibition of electrical activity in scorpion by norepinephrine. In line with this Madhusudhana (1995) reported a decrease in the spontaneous electrical activity in the scorpion *Heterometrus fulvipes* at a concentration of $1x10^{-3}$ M. This observation coincides with the decrease observed in the present study at concentrations of norepinephrine higher than $1x10^{-8}$ M. Thus there seems to be a concentration-dependent activation of spontaneous activity by NEP wherein $1x10^{-8}$ M elevates the activity significantly, and concentrations higher than this cause progressively lesser activation.

Epinephrine

Epinephrine (EP) like NEP, also elevated the spontaneous activity in the present investigation. However, the maximum elevation was achieved at a much higher concentration with EP, i.e. at $1x10^{-5}$ M, against the maximum elevation at $1x10^{-8}$ M itself with NEP (Plate-III; Table-13). This apparently

casts doubts on its possible transmitter role compared to NEP. In support of this, EP was not detected either in the nervous system or in the haemolymph throughout the 5th instar (see Chapter-II), indicating its non-conversion from NEP. However, the response to EP at higher concentrations resulting in a change in spontaneous activity indicates the presence of membrane reactivity to this compound. Thus, EP probably exercises its action on the silkworm nervous system much in the same way as any other non-transmitter chemical compound. Madhusudhana (1995) recorded a decrease in spontaneous activity of the ventral nerve cord of the scorpion *Heterometrus fulvipes* using $1x10^{-3}$ M EP, which is a logical consequence with high concentrations, as also noticed in the present study with concentrations higher than $1x10^{-5}$ M.

Epinephrine, nevertheless, was shown to cause significant effects either in the nervous system or its innervated organs in various other invertebrates including insects. It augments the rhythmic concentrations of wing muscles mediated by the metathoracic ganglion in *Locusta* [Voskresenskaya 1950]. It increases the heartbeat in the scorpion *Palamnacus bengalensis* [Kanungo 1957]. It has a stimulatory effect on the neurally mediated hindgut contractions in the crab [Florey 1954]. EP along with NEP and dopamine has also been projected as a neurotransmitter in vertebrates, subserving functions such as emotion, attention and visceral regulation [Coyle and Snyder, 1981]. However, the concentration of EP in the central nervous system is known to be relatively low, approximately 5 to 17% of the NEP content, implicating a relatively less important role as a neurotransmitter compared to NEP [Cooper, Bloom and Roth 1982].

Dopamine

Dopamine (DA) also caused an elevation in the spontaneous activity in the present study (Plate-V; Table-15). Maximum elevation was recorded at $1x10^{-6}$ M, with decreases at concentrations higher and lower than this. DA is an important chemical compound which serves a dual function, viz., as a precursor for nor-epinephrine and as a neurotransmitter itself. In most mammals it represents 50% of the total catecholamine content of the CNS [Cooper, Bloom and Roth 1982]. In the present study DA was detected on all the days of the 5th instar, both in the nervous system and haemolymph, as against NEP which was detected from the 5th day and EP which not detected at all (see Chapter-II). This probably indicates a more significant role for DA, and it could be a transmitter candidate in silkworm nervous system.

Using a high concentration of $1x10^{-3}$ M DA, Madhusudhana (1995) elicited a strong inhibitory action on the spontaneous activity in the ventral nerve cord of the scorpion *Heterometrus fulvipes*. A similar inhibitory action of DA was reported in the nervous system of the scorpion by Goyffon (1978). DA-induced inhibition was reported in other arthropods such as the stick insect [Dieter and Dorn 1992] and *Limulus* [James and Lent 1992]. In contrast to this, Janet and Ritzmann (1992) notice an increase in the amplitude of giant neuron-evoked excitatory postsynaptic potentials in the cockroach, *Periplaneta americana*. This report is in coherence with the excitation recorded with DA in the present study. Since excitation or inhibition is more a tissue response depending on the nature of receptors that may be depolarized or hyerpolarized by a particular chemical compound [O'Brien 1979], it is safe to assume that the reactivity of the receptors to DA in the nervous system of *Bombyx mori* is such as to result in excitation.

Serotonin (5-Hydroxytryptamine, 5-HT)

5-HT in the present study caused elevation in spontaneous activity, with maximum increase occurring with $1x10^{-8}$ M, following which the elevation was lowered at higher concentrations (Plate-VI; Table-16). The available literature on different invertebrates and vertebrates, however, suggests both excitation and inhibitory actions for 5-HT. At a high concentration of $1x10^{-3}$ M it produced a marked inhibition in the firing rate as well as amplitude of small spikes in the spontaneous activity of the ventral nerve cord of the scorpion, *Heterometrus fulvipes* [Madhusudhana 1995]. A similar inhibition due to serotonin in the spontaneous activity of prosonian nervous system of the scorpion *Androctonus maritanicus* was reported by Goyffon (1978). Inhibitory effect of serotonin was also reported by Hiroshi and Tanaka (1992) in the thoracic ganglion of cockroach. Janet and Ritzman (1992) reported inhibitory action of serotonin on the giant interneuron-evoked excitatory postsynaptic potentials in the cockroach. In vertebrates, 5-HT has been tested on cells that exhibit spontaneous electrical activity, and the majority of cells decreased their discharge rate in certain brain areas. However, in certain other regions, 5-HT caused pronounced activation and discharge rate. Therefore it was concluded that 5-HT causes mainly, if not exclusively, inhibitory effects [Cooper, Bloom and Roth 1982].

Several instances are also present where 5-HT caused pronounced elevatory effects. It was found to potentiate the excitatory action of glutamate in crustaceans [Robbins 1959]. It caused depolarization of resting potentials of tegumental and muscle cells and also increased the discharge frequency of

muscle cells in *Schistosoma japonicum* [Wang *et al.* 1995]. It caused an increase in the frequency and amplitude of spontaneous contractions in the trematode *Haplometra cylindracea* [McKay *et al.* 1989]. 5-HT had a high excitatory effect on the electrical activity of the digenean parasite *Cercaria caribbra* [Young *et al.* 1988]. In the honeybee, 5-HT increased the excitability of chemoreceptors and electrical activity of the 2nd thoracic ganglion at lower concentrations, while causing reduction at higher concentration [Lopatina *et al.* 1982].

Thus, all the biogenic amines examined in the present study caused elevation of spontaneous activity of the VNC at lower concentrations, while causing a decrease at higher concentrations, showing a dose-dependent effect. The concentration at which maximum elevation was caused varied from compound to compound. The elevation itself could be recorded in two ways, viz., (i) an increase in the overall frequency or the spikes, and (ii) an increase in the firing of spikes with larger amplitudes. The latter could be visualized through an increase in the number of spike categories. In all cases the spike categories were also maximum when the spike frequency was maximum. It is possible that some of the compounds examined in the present study for their effects on spontaneous activity could be acting as neurotransmitters in *Bombyx mori* besides subserving other functions. Epinephrine may be exempted from this possibility, since it was neither detected in the nervous system nor in haemolymph throughout the 5th instar, although it did cause an elevation in spontaneous activity.

Dopamine as the precursor for norepinephrine was present in the nervous system and haemolymph throughout, and could very well be a transmitter candidate. Norepinephrine made its appearance in the nervous system on the 5th day and was present during the rest of the instar, but was present in the haemolymph throughout. It is interesting that NEP appeared just three days before spinning, giving room for speculation on its role as a neurotransmitter in spinning activity. Serotonin in contrast was present in the nervous system throughout but appeared in the haemolymph from the 4th day only. The reason for its release into the haemolymph during the second half of the instar, four days before the spinning, is not clear, although one may speculate feedback actions on the nervous system by chemicals (neurohormones, neurohumours etc.) released by itself. Instances for such actions are available in literature on other invertebrates [Rao and Gopalakrishna Reddy 1967; Venkatachari 1971; Vijayalakshmi *et al.* 1977; Rajarami Reddy *et al.* 1978].

Compounds such as acetylcholine, glutamate, GABA, dopamine, norepinephrine, epinephrine, 5-hydroxytryptamine, histamine etc. have multifarious functions. They have demonstrated electrophysiological activity and thereby modulate the active state of the neuron and transmission of impulses. Changes in the concentrations of these substances in the nervous system have been shown to correlate with a change in the behavioural state of the animal [Cooper, Bloom and Roth 1982]. It is therefore possible that the changes in the levels of biogenic amines observed in the present investigation, and their elevatory effects on the spontaneous electrical activity of the nervous system may well be an indication as to the positive role they might have in the overall activity of the silkworm during metamorphosis, especially during the feeding and spinning phases of the 5th instar. This would be a productive avenue for further research.

LEGEND FOR PLATE - I

Oscilloscopic recordings showing the spontaneous electrical activity from different segments of the ventral nerve cord of *Bombyx mori* on the 1st day of the 5th instar.

A : Between the suboesophageal ganglion & 1st thoracic ganglion (SG-1TG).

B : Between the 1st thoracic ganglion & 2nd thoracic ganglion (1TG-2TG).

C : Between the 2nd thoracic ganglion & 3rd thoracic ganglion (2TG-3TG).

D : Between the 3rd thoracic ganglion & 1st abdominal ganglion (3TG-1AG).

E : Between the 1st abdominal ganglion & 2nd abdominal ganglion (1AG-2AG).

F : Between the 2nd abdominal ganglion & 3rd abdominal ganglion (2AG-3AG).

G : Between the 6th abdominal ganglion & 7/8 abdominal ganglion (6AG-7/8AG).

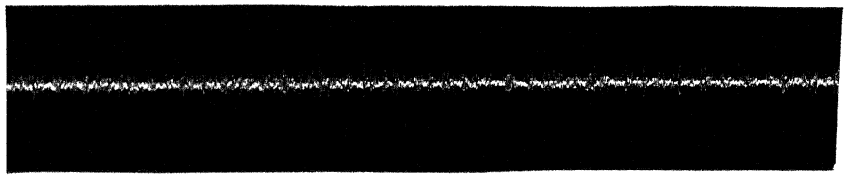
Calibration

Vertical : 1 Cm = 50 μv
Horizontal : 1 Cm = 100 ms

Note : Recordings from 3AG-4AG, 4AG-5AG and 5AG-6AG are not presented to avoid redundancy of the same level of activity of these abdominal segments as of 2AG-3AG and 6AG-7/8 AG.

PLATE - I

A



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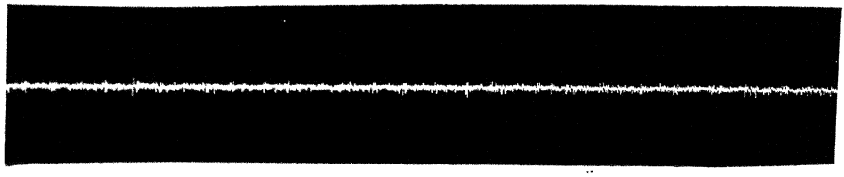

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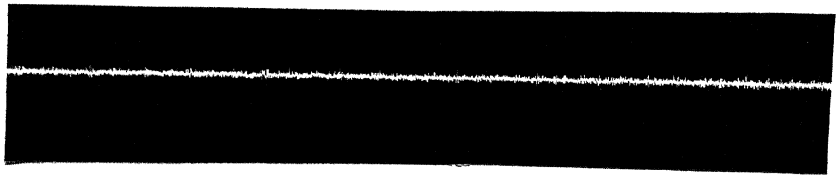

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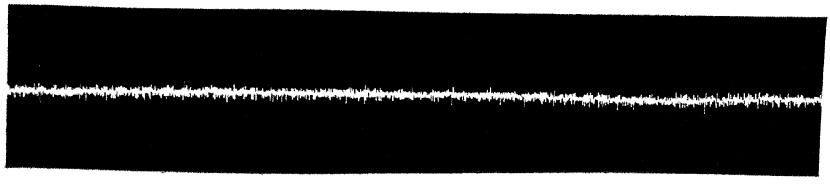

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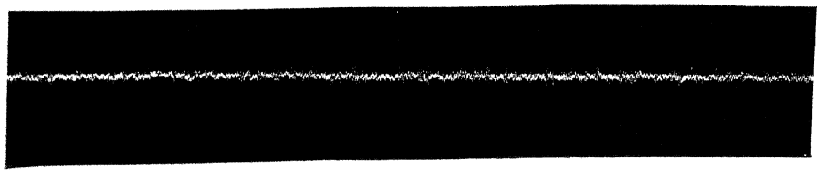

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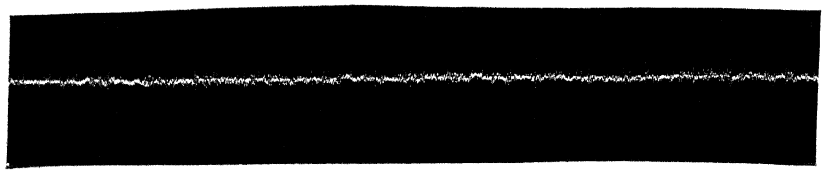

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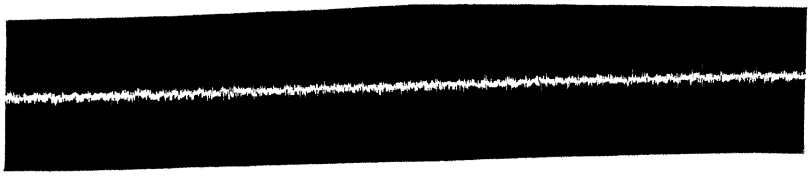


Table-11 : Spontaneous elecrical activity (spikes/sec) in different segments of the ventral nerve cord (VNC) of *Bombyx mori* on the **1st day of the 5th instar**. The activity is the mean ± standard deviation (SD) of six separate experiments.

Sl.No.	Segment of VNC		Number of Spikes	Number of Spike Categories
1.	SG - 1TG	Mean SD	212 ± 12	7
2.	1TG - 2TG	Mean SD	70 ± 7	3
3.	2TG - 3TG	Mean SD	88 ± 16	3
4.	3TG - 1AG	Mean SD	125 ± 17	4
5.	1AG - 2AG	Mean SD	164 ± 10	6
6.	2AG - 3AG	Mean SD	154 ± 8	6
7.	6AG - 7/8AG	Mean SD	155 ± 12	4

LEGEND FOR PLATE - II

Oscilloscopic recordings showing the spontaneous electrical activity from different segments of the ventral nerve cord of *Bombyx mori* on the 7th day of the 5th instar.

A : Between the suboesophageal ganglion & 1st thoracic ganglion (SG-1TG).

B : Between the 1st thoracic ganglion & 2nd thoracic ganglion (1TG-2TG).

C : Between the 2nd thoracic ganglion & 3rd thoracic ganglion (2TG-3TG).

D : Between the 3rd thoracic ganglion & 1st abdominal ganglion (3TG-1AG).

E : Between the 1st abdominal ganglion & 2nd abdominal ganglion (1AG-2AG).

F : Between the 2nd abdominal ganglion & 3rd abdominal ganglion (2AG-3AG).

G : Between the 6th abdominal ganglion & 7/8 abdominal ganglion (6AG-7/8AG).

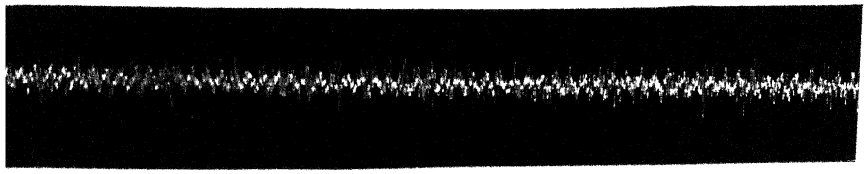
Calibration

Vertical : 1 Cm = 50 µv
Horizontal : 1 Cm = 100 msec.

Note : Recordings from 3AG-4AG, 4AG-5AG and 5AG-6AG are not presented to avoid redundancy of the same level of activity of these abdominal segments as of 2AG-3AG and 6AG-7/8 AG.

PLATE - II

A



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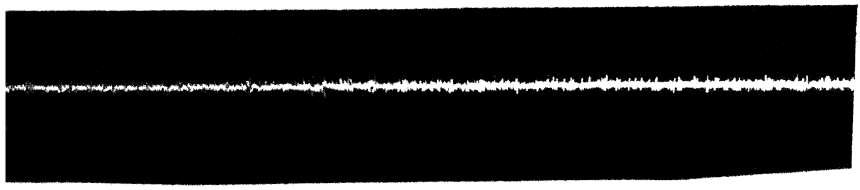

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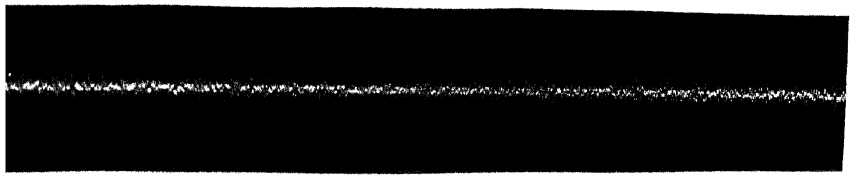

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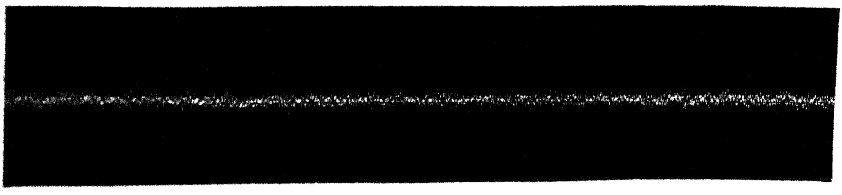

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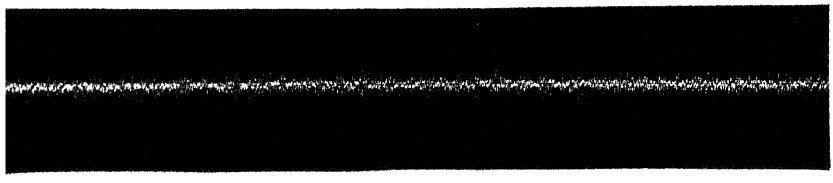

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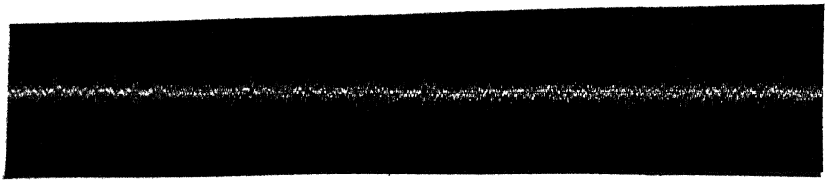


G

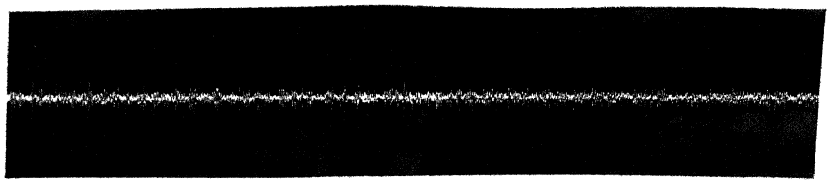


Table-12 : Spontaneous elecrical activity (spikes/sec) in different segments of the ventral nerve cord (VNC) of *Bombyx mori* on the **7th day of the 5th instar**. The activity is the mean ± standard deviation (SD) of six separate experiments.

Sl.No.	Segment of VNC		Number of Spikes	Number of Spike Categories
1.	SG - 1TG	Mean SD	275 ± 18	9
2.	1TG - 2TG	Mean SD	103 ± 14	4
3.	2TG - 3TG	Mean SD	157 ± 13	7
4.	3TG - 1AG	Mean SD	162 ± 8	5
5.	1AG - 2AG	Mean SD	241 ± 13	6
6.	2AG - 3AG	Mean SD	228 ± 9	6
7.	6AG - 7/8AG	Mean SD	245 ± 10	8

LEGEND FOR PLATE - III

Oscilloscopic recordings showing the effect of **epinephrine** (EP) at different concentrations from $1x10^{-7}$ M to $1x10^{-4}$ M on the spontaneous electrical activity from the ventral nerve cord between the 2nd and 3rd abdominal ganglia of *Bombyx mori* on the 7th day of the 5th instar.

A : Control recording

B : Recording on treatment with $1x10^{-7}$M EP.

C : Recording on treatment with $1x10^{-6}$M EP.

D : Recording on treatment with $1x10^{-5}$M EP.

E : Recording on treatment with $1x10^{-4}$M EP.

F : Recording on treatment with $1x10^{-4}$M EP.

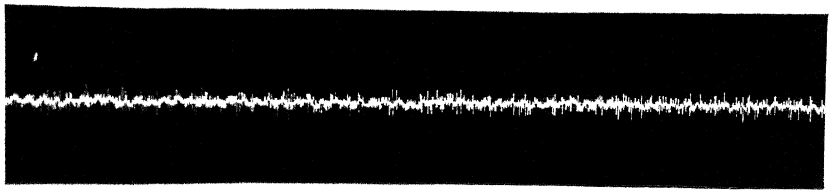
For each treatment the nerve cord was soaked in the test medium for 3 min before recording the activity, and then washed with Ringer thoroughly for 5 min before the cord was treated with the next higher concentration. The recordings of reversal of activity to control on washing are not shown, since at all the above concentrations of EP, the effect could be reversed to the control by washing with Ringer.

Calibration

Vertical : 1 Cm = 50 μv
Horizontal : 1 Cm = 100 msec.

PLATE - III

A



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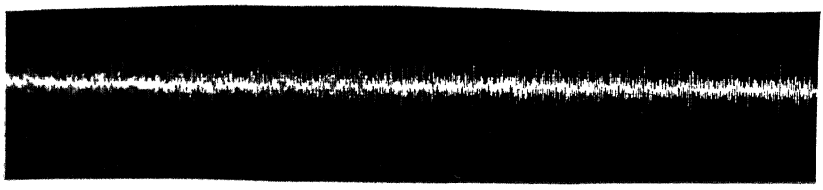

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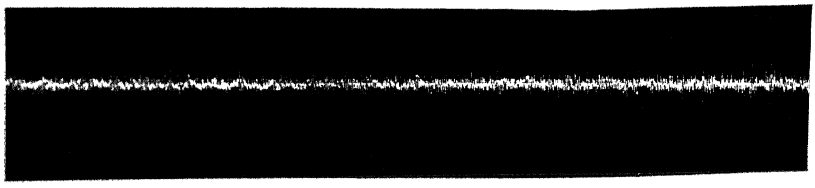

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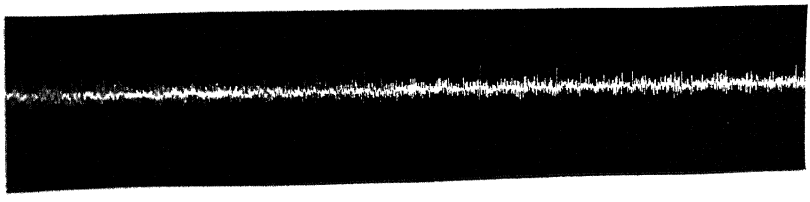

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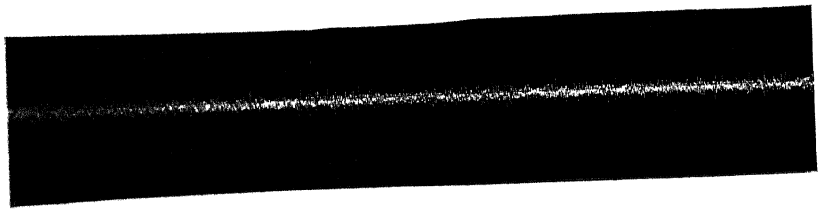


F

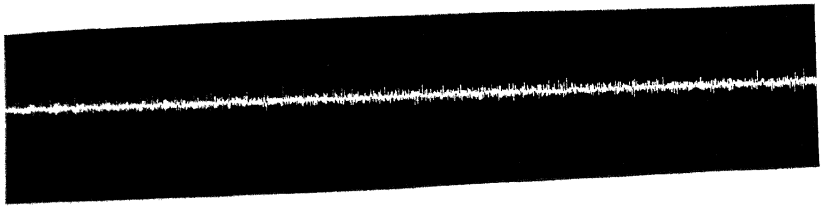


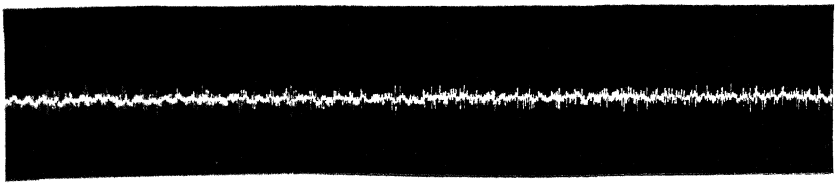
Table-13 : Effects of **epinephrine (EP)** at different concentrations ($1x10^{-8}$ M to $1x10^{-4}$ M) on the spontaneous electrical activity (spikes/sec) of the ventral nerve cord (VNC) in 7th day larva of the 5th instar of *Bombyx mori*. The activity is the mean ± standard deviation (SD) of six separate experiments.

Sl.No.	Nature of Treatment		Number of Spikes	Number of Spike Categories
1.	Ringer (Control)	Mean SD	213 ± 14	5
2.	$1x10^{-8}$M	Mean SD % Change	243 ± 15 + 14.18*	6
3.	$1x10^{-7}$M	Mean SD % Change	285 ± 26 + 33.89***	7
4.	$1x10^{-6}$M	Mean SD % Change	298 ± 19 - 40.09***	8
5.	$1x10^{-5}$M	Mean SD % Change	340 ± 18 + 60.00***	8
6.	$1x10^{-4}$M	Mean SD % Change	216 ± 20 + 1.31NS	5

*** = P < 0.001; * = P < 0.05; NS: Not significant.

PLATE - IV

A



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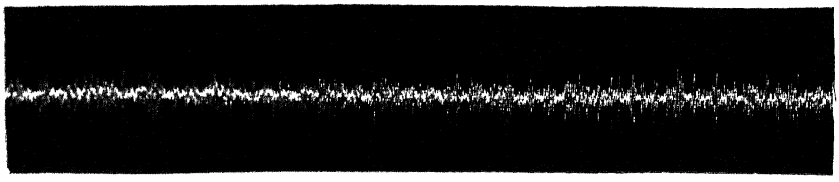

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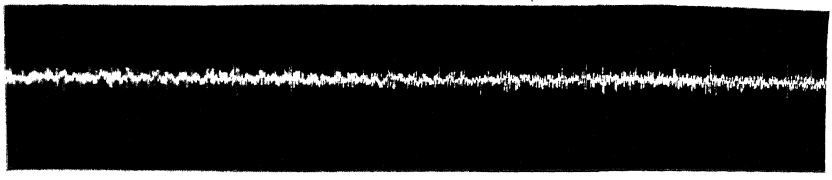

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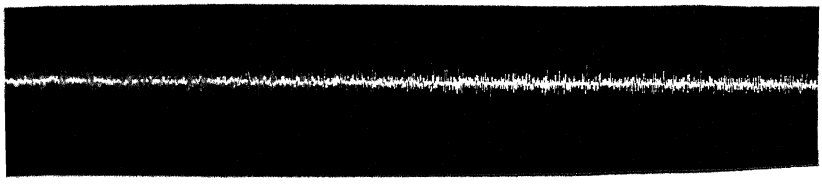

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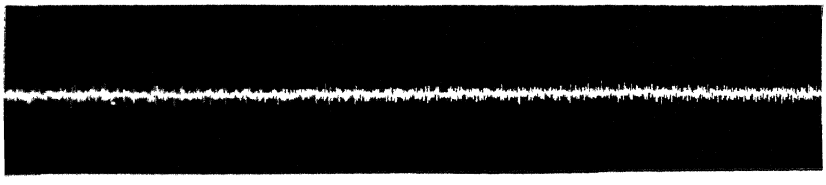


F

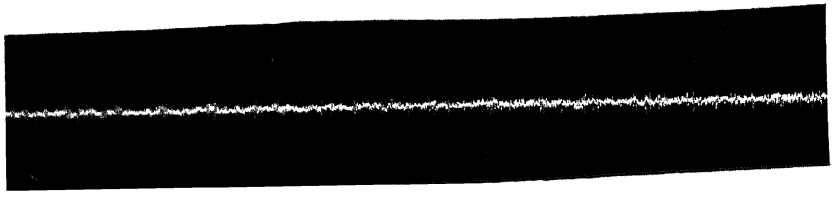


Table-14 : Effects of **nor-epinephrine (NEP)** at different concentrations ($1x10^{-7}$ M to $1x10^{-4}$ M) on the spontaneous electrical activity (spikes/sec) of the ventral nerve cord (VNC) in 7th day larva of the 5th instar of *Bombyx mori*. The activity is the mean ± standard deviation (SD) of six separate experiments.

Sl.No.	Nature of Treatment		Number of Spikes	Number of Spike Categories
1.	Ringer (Control)	Mean SD	210 ± 13	5
2.	$1x10^{-7}$ M	Mean SD % Change	443 ± 18 + 110.86***	11
3.	$1x10^{-6}$ M	Mean SD % Change	292 ± 16 + 39.24***	6
4.	$1x10^{-5}$ M	Mean SD % Change	275 ± 17 + 30.86***	6
5.	$1x10^{-4}$ M	Mean SD % Change	223 ± 13 + 6.38^{NS}	4
6.	$1x10^{-4}$M	Mean SD % Change	198 ± 14 + 5.62^{NS}	6

*** = $P < 0.001$; NS: Not significant.

LEGEND FOR PLATE - V

Oscilloscopic recordings showing the effect of **dopamine** (DA) at different concentrations from $1x10^{-8}$ M to $1x10^{-4}$ M on the spontaneous electrical activity from the ventral nerve cord between the 2nd and 3rd abdominal ganglia of *Bombyx mori* on the 7th day of the 5th instar.

A : Control recording

B : Recording on treatment with $1x10^{-8}$M DA.

C : Recording on treatment with $1x10^{-7}$M DA.

D : Recording on treatment with $1x10^{-6}$M DA.

E : Recording on treatment with $1x10^{-5}$M DA.

F : Recording on treatment with $1x10^{-4}$M DA.

For each treatment the nerve cord was soaked in the test medium for 3 min before recording the activity, and then washed with Ringer thoroughly for 5 min before the cord was treated with the next higher concentration. The

[illegible] shown, since

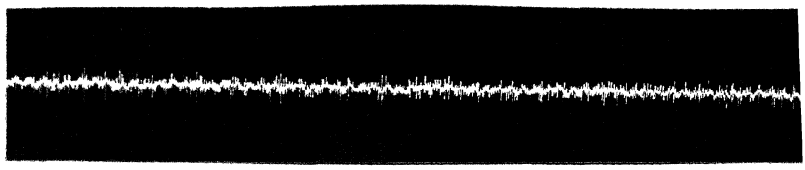
at all the above concentrations of DA, the effect could be reversed to the control by washing with Ringer.

Calibration

Vertical : 1 Cm = 50 µv
Horizontal : 1 Cm = 100 msec.

PLATE - V

A



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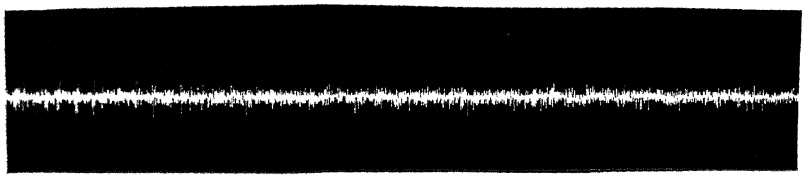
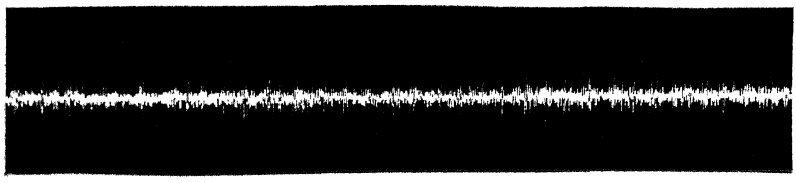
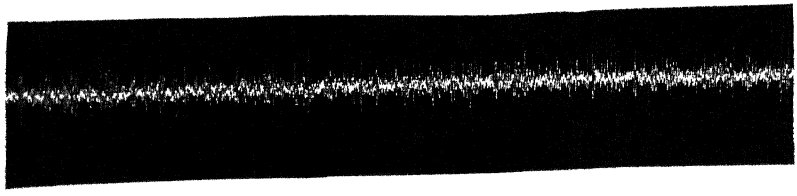


C



D



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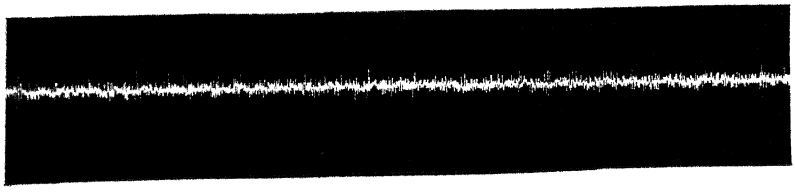

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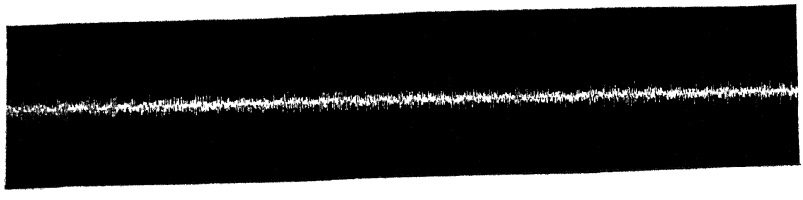


Table-15 : Effects of **dopamine (DA)** at different concentrations ($1x10^{-8}$ M to $1x10^{-4}$ M) on the spontaneous electrical activity (spikes/sec) of the ventral nerve cord (VNC) in 7th day larva of the 5th instar of *Bombyx mori*. The activity is the mean ± standard deviation (SD) of six separate experiments.

Sl.No.	Nature of Treatment		Number of Spikes	Number of Spike Categories
1.	Ringer (Control)	Mean SD	217 ± 21	5
2.	$1x10^{-8}$M	Mean SD % Change	248 ± 12 + 14.47*	6
3.	$1x10^{-7}$M	Mean SD % Change	265 ± 14 + 22.21***	8
4.	$1x10^{-6}$M	Mean SD % Change	314 ± 8 + 44.88***	9
5.	$1x10^{-5}$M	Mean SD % Change	259 ± 15 + 19.45***	5
6.	$1x10^{-4}$M	Mean SD % Change	204 ± 14 - 5.81NS	5

*** = P < 0.001; = P < 0.05; NS: Not significant.

LEGEND FOR PLATE - VI

Oscilloscopic recordings showing the effect of **5-hydroxy tryptamine** (5-HT) at different concentrations from $1x10^{-8}$ M to $1x10^{-4}$ M on the spontaneous electrical activity from the ventral nerve cord between the 2nd and 3rd abdominal ganglia of *Bombyx mori* on the 7th day of the 5th instar.

A : Control recording

B : Recording on treatment with $1x10^{-8}$M 5-HT.

C : Recording on treatment with $1x10^{-7}$M 5-HT.

D : Recording on treatment with $1x10^{-6}$M 5-HT.

E : Recording on treatment with $1x10^{-5}$M 5-HT.

F : Recording on treatment with $1x10^{-4}$M 5-HT.

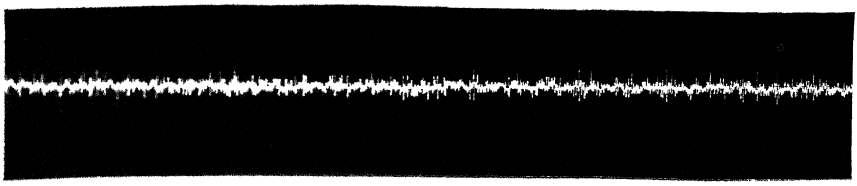
For each treatment the nerve cord was soaked in the test medium for 3 min before recording the activity, and then washed with Ringer thoroughly for 5 min before the cord was treated with the next higher concentration. The recordings of reversal of activity to control on washing are not shown, since at all the above concentrations of 5-HT, the effect could be reversed to the control by washing with Ringer.

Calibration

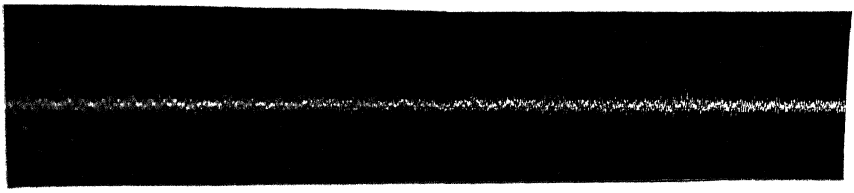
Vertical : 1 Cm = 50 μv
Horizontal : 1 Cm = 100 msec.

PLATE - VI

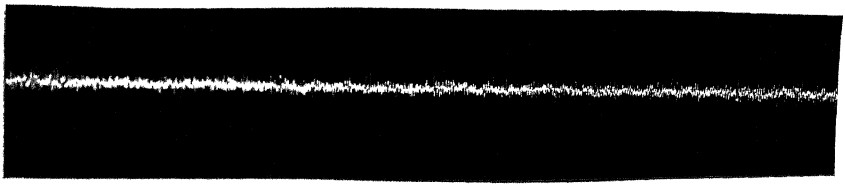
A



B



C



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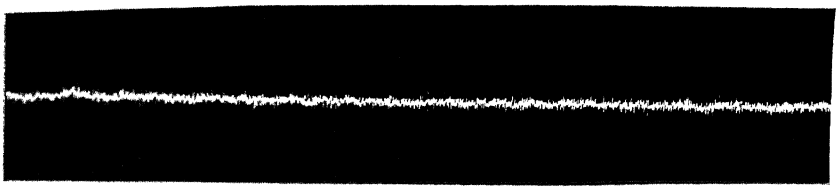

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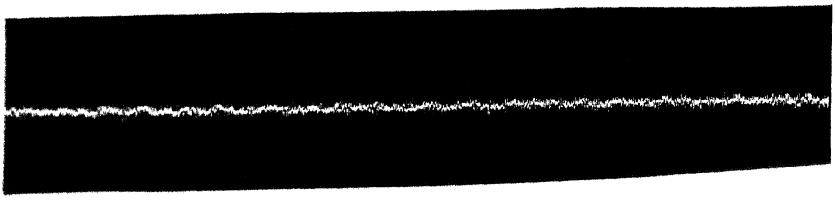

F

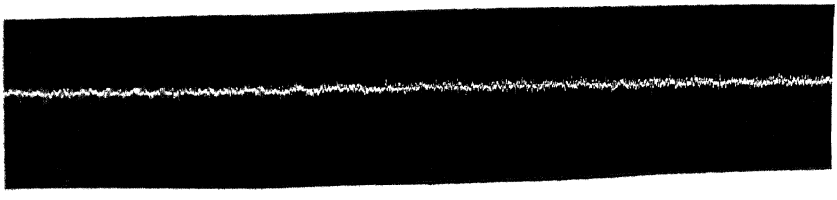

Table-16 : Effects of **5-hydroxytryptamine (5-HT)** at different concentrations ($1x10^{-8}$ M to $1x10^{-4}$ M) on the spontaneous electrical activity (spikes/sec) of the ventral nerve cord (VNC) in 7th day larva of the 5th instar of *Bombyx mori*. The activity is the mean ± standard deviation (SD) of six separate experiments.

Sl.No.	Nature of Treatment		Number of Spikes	Number of Spike Categories
1.	Ringer (Control)	Mean SD	214 ± 16	5
2.	$1x10^{-8}$M	Mean SD % Change	324 ± 13 + 51.45***	6
3.	$1x10^{-7}$M	Mean SD % Change	289 ± 20 + 34.83***	6
4.	$1x10^{-6}$M	Mean SD % Change	277 ± 12 + 29.13***	4
5.	$1x10^{-5}$M	Mean SD % Change	266 ± 10 + 24.37***	4
6.	$1x10^{-4}$M	Mean SD % Change	233 ± 10 + 8.96*	4

*** = P < 0.001; * = P < 0.05.

Summary and Conclusions

1. The present study was aimed at examining the day-to-day changes in certain parameters of carbohydrate metabolism, levels of certain neurotransmitters, as well as the electrical activity of the nervous system during 5th instar of the silkworm *Bombyx mori*.

2. PM X NB_4D_2 (Multivoltine x Bivoltine) hybrid variety of *Bombyx mori* was selected for the investigations.

3. The silkworms were reared following standard procedures. They were fed with M_5 variety of mulberry leaves.

4. The biochemical parameters examined were total carbohydrates, glycogen, glycogen phosphorylase, glucose, trehalose and trehalase.

5. The total carbohydrate level showed an increase from the 1st day to the 9th day of the 5th instar and thereafter decreased up to the 1st day of the pupa. The trend was similar in all the tissues studied.

6. The increase in total carbohydrates could be attributed to the need for energy during growth and development of the silkworm. Larvae of the 5th instar consume enormous quantity of leaf, which could lead to higher levels of carbohydrates in the 5th instar. The decreasing levels during spinning period could be due to cessation of feeding, and to their mobilization on higher energy demand for spinning.

7. The levels of glycogen in all the tissues increased from the 1st day to the 6th day of the 5th instar and thereafter declined on the 7th day

and also on the 1st day of the pupa. The increasing levels of glycogen are indicative of increased glycogenesis during the feeding periods. Similarly their decrease during the non-feeding periods is reflective of elevation of glycogenolysis to meet the energy demands.

8. The phosphorylase 'a' activity was higher at the beginning of the 5th instar and then decreased during the middle. Thereafter it showed elevated levels towards the end of the 5th instar and also on the 1st day of the pupa. Phosphorylase 'b' showed an opposite trend. Higher activity levels of phosphorylase 'a' reflect the decrease in glycogen content and mobilization of glycogen as a source of energy. The decrease during the middle of the 5th instar suggests lowered mobilization of glycogen. In contrast to phosphorylase 'a', the lowest level of phosphorylase 'b' during the early and late stage of the 5th instar and also on the 1st day of the pupa indicates that the phosphorylase 'b' is probably converted into the 'a' form.

9. The glucose levels in all the tissues increased from the 1st day to the 7th day of the 5th instar and then decreased on the 1st day of the pupa. These changes clearly indicate its build-up during the early periods for its utilization later for energy for the active spinning phase.

10. The levels of trehalose also showed a similar trend as that of glucose in all the tissues. High content of trehalose indicates the energy demand of the insect. Further there is a progressive increase in the trehalose content from the 1st day to the 7th day of the 5th instar.

Trehalose is a major carbohydrate and important reserve metabolite in insects. The decrease on the 1st day of pupa in all tissues might be due to the mobilization of trehalose into the energy stores for utilization during the pupal and adult life.

11. The activity levels of trehalase are higher in the early and late stages of the 5th instar and also on the 1st day of the pupa, while the middle of the 5th instar showed lower activity levels. Higher activity of trehalase could meet the energy demands and by breaking down trehalose in the tissues. The decrease in the trehalase activity coincides with the accumulation of trehalose during the middle of the 5th instar. Accumulation of trehalose seems to match the demand for its breakdown for energy.

12. Nor-epinephrine (NEP) was not detected in the CNS in early stages of the 5th instar. It only appeared on the 5th day and thereafter increased up to the 1st day of the pupa. In the haemolymph, NEP was present from the 1st day throughout the instar. It was higher in the beginning of the 5th instar and thereafter showed a decreasing trend. It increased again towards the end of the 5th instar and also on the 1st day of the pupa.

13. Dopamine increased from 1st day to the 7th day of the 5th instar and also on the 1st day of the pupa in the nervous system. However, in the haemolymph DA increased from the 1st day to the 7th day of the pupa.

14. The concentration of 5-hydroxytryptamine (serotonin) showed an increasing trend during the 5th instar and decreased on the 1st day of the pupa. In the haemolymph 5-HT was not detected in the early stages of the 5th instar. It appeared only from the 4th day of the instar and thereafter increased up to the 1st day of the pupa. The low values in haemolymph indicate relatively low release or diffusion from the tissues into the haemolymph.

15. Spontaneous electrical activity recorded from different segments of the ventral nerve cord differed in the frequency and number of spike categories firing. The activity was highest between the suboesophageal ganglion and 1st thoracic ganglion. Lower activity was recorded in the thoracic part of the cord and the activity increased in the abdominal cord. More or less the same level of activity was recorded from all the abdominal segments of the cord. The above trend was the same from the 1st day to the 7th day of the 5th instar. However, the overall level of activity increased from the 1st day to the 7th day in tune with the increasing complexity of the peripheral nervous system in the silkworm from the 1st day to the 7th day of the 5th instar.

16. Treatment of the cord with solutions of neurotransmitter substances such as epinephrine (EP), norepinephrine (NEP), dopamine (DA) and

5-hydroxytryptamine (5-HT) at different concentrations from $1x10^{-8}$ M to $1x10^{-4}$ M showed elevation at lower concentrations and decrease at $1x10^{-4}$ M. The concentration at which maximum elevation could be elicited varied between the above transmitters. The possibility remains that one or more of these substances could act as neurotransmitters in the silkworm nervous system.

The present investigation reveals that the carbohydrate metabolism is geared up to meet the demands for energy from the 1st day to the 7th day of the 5th instar of the silkworm *Bombyx mori*. The electrical activity also shows an increase from the 1st day to the 7th day of the instar in tune with the increase in the complexity of the nervous system. One or more of the different neurotrasmitters may be playing a role in modulating the activity of the nervous system during the progression of the 5th instar, and during the spinning activity of the silkworm towards building the cocoon.

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